Function of *ALS* Genes of *Candida albicans* in Catheter Adhesion

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摘要

導管是一種插入人體中用於靜脈注射液體，藥物和體液引流的一種醫療設備。最常用的導管是中央靜脈導管，周邊靜脈導管和導尿管。導管的使用經常導致與導管相關的感染。最常被鑒別的導管相關感染的致病源包括凝固酶陰性葡萄球菌如表皮葡萄球菌，金黃色葡萄球菌，大腸桿菌，腸桿菌屬和白色念珠菌。白色念珠菌是一種機會性感染致病菌，它感染免疫功能降低的病人。這些病人的免疫系統受到損壞，所以他們有著較高的獲得導管相關感染的風險。與白色念珠菌感染相關的導管能導致散播性念珠菌感染。 對導管相關感染的致病機理的理解有助於建立預防和治療策略。 粘附是感染的第一步。 白色念珠菌的粘附能力越強致病能力越高。

白色念珠菌的類凝集素序列基因家族包括 8 個成員(*ALS1*, *ALS2*, *ALS3/ALS8*, *ALS4*, *ALS5*, *ALS6*, *ALS7*, and *ALS9*)。 *ALS1*, *ALS3* 和 *ALS5* 的基因產物表明它們有助於白色念珠菌粘附內皮和 FaDu 外皮細胞上。對白色念珠菌粘附分子因素的理解有助於提高我們對導管相關感染的認識。這篇論文研究的目的是（i）研究接種量對導管粘附的影響以及建立一個粘附模型的定量系統和（ii）研究 *ALS1*, *ALS5* 小等位基因和 *ALS6* 轉化到釀酒酵母中以及該釀酒酵母粘附于人類纖維細胞，乙烯丙烯氟化物導管，聚胺基甲酸酯導管以及矽酮導管的影響。

接種量效果實驗表明當接種量爲 $1x10^5$, $1x10^6$, $1x10^7$ 個細胞時，白色念珠菌對上述三種導管的粘附能力是沒有分別的。粘附模型定量系統使用三磷酸腺生物

發光檢測技術。其線性範圍爲 $1x10^3$-$1x10^7$ 個細胞。 其檢測極限爲 $1x10^4$ 個細胞。 *ALS1*, *ALS5* 小等位基因和 *ALS6* 的轉化有助於釀酒酵母粘附于人類纖維細胞。 *ALS1* 和 *ALS6* 的轉化有助於釀酒酵母粘附於乙烯丙烯氟化物導管但不能使釀酒酵母粘附於聚胺基甲酸酯導管以及矽酮導管。*ALS5* 小等位基因的轉化不能使釀酒酵母粘附於上述 3 種導管。

這研究得到以下結論: 當接種量爲 $1x10^5$ 致 $1x10^7$ 個細胞時，白色念珠菌對乙烯丙烯氟化物導管，聚胺基甲酸酯導管以及矽酮導管的粘附能力不受接種菌量影響。當接種量爲 $1x10^7$ 個細胞時, 三磷酸腺生物發光檢測技術達致研究粘附起始階段所需的敏感度。 *ALS1*, *ALS5* 細等位基因和 *ALS6* 有助粘附人類纖維細胞。 *ALS* 基因對乙烯丙烯氟化物導管顯現不同的粘附力, 但對其他導管沒有粘附力。

Abstract

Catheter is a medical device inserted into body for administration of intravenous fluid, medication or drainage of body fluid etc. Central venous catheters (CVC), peripheral venous catheters (PVC), and urinary catheters are the commonly used catheters. The uses of catheters often lead to catheter associated infection (CAI). The most frequently identified causative agents of CAI include coagulase negative *Staphylococci* such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter* species, and *Candida albicans*. *Candida albicans* is an opportunistic pathogen infecting immunocompromised patients. The immune system of these patients is impaired and therefore they have a higher risk of acquiring CAI. Catheter associated infections related to *Candida albicans* can result in disseminated candidiasis. Understanding of the pathogenesis of CAI of *Candida albicans* helps to develop strategy for the prevention and treatment of CAI. Adhesion is the initial step to establish an infection. It was demonstrated that the more adhesive the *Candida albicans* is, the more pathogenic it is. Agglutinin-like sequence (*ALS*) gene family of *Candida albicans* includes 8 members (*ALS1*, *ALS2*, *ALS3/ALS8*, *ALS4*, *ALS5*, *ALS6*, *ALS7*, and *ALS9*). Gene products of *ALS1, ALS3* and *ALS5* were shown to contribute to the adhesion of *Candida albicans* to endothelial cells and FaDU epithelial cells. Understanding the molecular factors contributing to adhesion of *Candida albicans*

may help to improve our knowledge in CAI. The aim of this study is (i) to study the effect of inoculum size in an adhesion model of catheters and to establish a quantitation system for the adhesion model; and (ii) to study the effect of transformation of *ALS1, ALS5* smaller allele, and *ALS6* to *Saccharomyces cerevisiae* on its adhesion to human fibroblasts, and to tetrafluoroethylene and hexafloropropylene polymer (FEP), polyethylene, and silicone catheters.

The study of effect of inoculum size revealed that adhesion of *Candida albicans* cells to the three catheters were not affected by the inoculum size of $1x10^5$, $1x10^6$, and $1x10^7$ cells. An ATP bioluminescence assay was established as the quantitation system of the adhesion model of catheters. The linear range was $1x10^3$ to $1x10^7$cells. The detection limit was $1x10^4$ cells. Transformation of *ALS1, ALS5* smaller allele, and *ALS6* conferred *Saccharomyces cerevisiae* adherence to human fibroblasts. Transformation of *ALS1*, and *ALS6* conferred *Saccharomyces cerevisiae* adherence to FEP catheter fragments but not to polyurethane, and silicone catheter fragments. Transformation of *ALS5* smaller allele did not confer *Saccharomyces cerevisiae* adherence to the three catheter fragments.

It was concluded that adherence of *Candida albicans* to FEP, polyurethane, and silicone catheter was not affected by initial inoculum size of *Candida albicans* cells in the range from $1x10^5$ cells to $1x10^7$ cells. The ATP bioluminescence used in

this study was sensitive enough for adhesion assay aim at studying initial adhesion events when initial inoculum size was $1x10^7$ cells. *ALS1*, *ALS5* smaller allele, and *ALS6* contributed to adherence to human fibroblasts. These *ALS* genes contributed to differential adherence to FEP catheters but not to other catheters.

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List of Abbreviations

Ala	Alanine
ALS	Agglutinin like sequence
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
blastn	Nucleotide-nucleotide blast program
CAI	Catheter associated infection
cDNA	Coding DNA
CFU	Colony forming unit
CO_2	Carbon dioxide
CoNS	Coagulase negative *Staphylococci*
CVCAI	Central venous catheter associated infection
CVCBSI	Central venous catheter associated bloodstream infection
DLVO theory	Derjaguin, Landau, Verwey and Overbeek theory
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EPIC	European Prevalence of Infection in Intensive Care
FEP	Tetrafluoroethylene and hexafloropropylene polymer
GPI	Glycosylphosphatidylinositol
ICU	Intensive care unit
Leu	Leucine
MATH	Microbial adhesion to hydrocarbon test
MEM	Minimum essential medium
MRS	Major repeat sequence
NCBI	National Center for Biotechnology Information
NNIS	National Nosocomial Infections Surveillance System
OD	Optical density
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.05% tween-20
PBSTM	PBST with 5% nonfat, dry milk
PCR	Polymerase chain reaction
Phe	Phenylalanine
PMSF	Phenylmethysulfunyl fluoride
PVC	Peripheral venous catheter

PVCAI	Peripheral venous catheter associated infection
PVDF	Polyvinylidene difluoride
rcf	Relative centrifugal force
RH	Relative humidity
RLU	Relative luminescence unit
RNA	Ribonucleic acid
r.p.m.	Round per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SAA	Slime associated antigen
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulfate
SSU rDNA	Small subunit ribosomal DNA
TBE	Tris-borate EDTA
Thr	Threonine
TPN	Total parenteral nutrition
UCAI	Urinary catheter associated infection
YPD	Yeast peptone dextrose
YPDB	Yeast peptone dextrose agar with blasticidin

Chapter 1 Introduction

1.1 Epidemiology of catheter associated infections

1.1.1 Catheter associated infections (CAI)

Catheters are widely used in health care settings. Intravascular catheters are used in the administration of blood products, total parenteral nutrition (TPN), fluid and electrolytes, and drugs (1). They are also used for hemodialysis (2), monitoring of hemodynamics status in intensive care unit (ICU) (3). They are also used for the drainage of cerebrospinal fluids to control the cerebrospinal fluid pressure in brain or spinal cord, and drainage of urine. However, use of catheters is often followed by secondary infections or nosocomial infections. These infections were named as CAI (4).

Clinical presentations of CAI vary, depending on the types of catheters used, the insertion site, and the duration of catheterization. Catheter associated infections can be categorized into local infections and systemic infections (5). Local CAI can be subclinical (5). Clinical presentations of local vascular CAI include inflammation, local pain, erythema, phlebitis, purulence and, in more serious cases, abscess, cellulitis, and septic thrombophlebitis (6). In systemic infections fever, bacteremia, leukocytosis, endocarditis, and dissemination of infection to bones, joints and other organs can be seen (5, 6). Fever may be absent in the elderly or

immunocompromised patients (6). Again, systemic infections can be subclinical (5).

There is no standard definition of CAI (7). Definitions served for surveillance purpose are different from those for clinical studies. The surveillance definitions for catheter associated bloodstream infections include all primary bloodstream infections in patients with an intravascular catheter when other sites of infection have been excluded. Primary bloodstream infection is defined as bacteremia or fungemia without documented distal source of infection (8). The clinical definitions for CAI include primary bloodstream infection with an intravascular catheter, presentation of fever, chills, and hypotension, no apparent site of infection in other source, and isolation of the same microorganism(s) from catheters as from the bloodstream (7, 9). As clinical definitions are more stringent, more bloodstream infections will be considered as catheter associated when the clinical definitions are used.

1.1.2 Risk and mortality of CAI

Catheterization was considered to be the most significant single risk factor of nosocomial infections in several studies (1, 7, 10, 11). According to National Nosocomial Infections Surveillance System (NNIS) of the United States of America, eighty seven percent of primary bloodstream infections were associated with the use of central line (10). In the European Prevalence of Infection in Intensive Care (EPIC)

study, the odd ratio of catheterization for bloodstream infections was 4.6, and the confidence interval was 3.1-6.8 (1). In another study, catheterization was found to be associated with 97% of urinary tract infections in ICU and the duration of catheterization was the most important risk factor for bacteriuria, ranging from 2.3-22.4 days (11). The attributable mortality of central venous catheters associated bloodstream infections (CVCBSI) ranged from 14%-28% in different studies (12, 13, 14). Central venous catheter associated bloodstream infections also resulted in a 6.5 days of excess ICU stay in the United States (1). Urinary catheter associated infections (UCAI) also led to a 1 day increase in hospitalization. The mortality rate of UCAI ranged from 14%-19% (11).

1.1.3 Etiology of CAI

The microorganisms causing CAI can be recovered by direct culture of catheters removed from patients (Maki's role method), and by molecular detection (15). Microorganisms can also be observed on the surface of catheters by microscopy (3). In one study, microorganisms were observed on the external catheter surfaces from all 41 catheters associated with bacteremia (3). It was found that the number of microorganisms present on the external catheter surfaces correlated with the percentage of catheters with purulence at the insertion site in a rabbit model experiment (18). These findings support the idea that the clinical presentations of CAI

are microbiological in nature, instead of irritation due to catheters or infusates as in the case of aseptic phlebitis (3) and trauma due to insertion of catheter (16).

The etiological microorganisms of CAI are numerous, depending on the catheter types, the insertion site, the purposes of the catheters, the nature of infusates, and the characteristics of patients.

1.1.3.1 Venous catheters

Several studies revealed that the first three etiological microorganisms in central venous catheter associated infections (CVCAI) are *Staphylococcus epidermidis* or coagulase negative *Staphylococcus* species (CoNS), *Staphylococcus aureus*, and *Candida* species (1, 17, 18, 19, 20). Coagulase negative *Staphylococci* were the leading cause of peripheral venous catheter associated infections (PVCAI) in several studies, responsible for 96.7% of the PVCAI of adults in one study (3). It was followed by *Staphylococcus aureus* and yeast. Each accounted for 1.6% of PVCAI. These three microorganisms accounted for 99.9% of PVCAI. In the bloodstream infections associated with pulmonary artery catheters, the first three causes were also CoNS (37%), *Staphylococcus aureus* (26%) and *Candida* species (16%). Enteric gram negative bacilli species were also a significant cause (11%) (20).

1.1.3.2 Urinary catheters

The etiological microorganisms in UCAI are significantly different from

those in CVCAI. *Escherichia coli* accounted for 26% of UCAI, Enterococci, 16%, *Pseudomonas aeruginosa*, 12%, *Candida* species, 9%, and *Klebsiella pneumoniae*, 6.4% (11). The etiological microorganisms of UCAI are different from CVCAI and PVCAI because the catheter insertion sites are different (2,11).

The etiological microorganisms were also influenced by the characteristics of patients. For example, the most frequently identified microorganisms in the CVCAI of burns patients was *Pseudomonas aeruginosa*, instead of *Staphylococcus epidermidis*. Finally, the incidence of CAI due to gram positive microorganisms and fungi increased significantly in the last two decades (1). This may be due to the increase in liver transplant recipients (21) and immunocompromised, neutropenic patients (22).

1.2 Pathogenesis of CAI

Catheter associated infections begin with the colonization of microorganism(s) on the originally sterile catheters. The microorganisms colonized on catheter surfaces, then grow and excrete extracellular matrix, resulting in the formation of a biofilm (23). Subsequently, microorganisms may be released into surrounding fluids such as bloodstream and urine, depending on the surrounding anatomical structures of the catheters. The clinical presentations and the final outcome of CAI are the results of the interactions between hosts (the immunity of the patients,

the underlying diseases etc), pathogens (the virulence of pathogens, the ability to form biofilm (23)) and the environment (the duration of catheterization, the technique of insertion, the insertion sites, the materials of the catheters inserted) (6).

1.2.1 Central venous catheters (CVC)

The main sources of microorganisms in CVCAI are normal flora from the surrounding skin, contaminated catheter hubs, contaminated infusates or disinfectants, hematogenous seedings, and contiguous infections (3, 17, 18, 24, 25). The source of colonizing microorganisms is related to the duration of catheterization. It was found that CVCs were first colonized by microorganisms from skin (average 5.1 days after insertion), then microorganisms from hematogenous seedings (average 8.6 days) and finally microorganisms from contaminated catheter hubs (average 13.1 days). The finding implicates different sources of microorganism for short term and long term CVCs. The microorganisms on the external surface of CVCs migrated to catheter tips by capillary action at the catheter-skin interface (25). Host proteins including albumins, fibronectins deposit on to the catheter tips surface almost immediately after insertion. Fibronectin, fibrin and fibrinogen can promote the adhesion of *Staphylococcus aureus*. Colonizing microorganisms accumulate and excrete extracelluar matrix such as slime or glycocalyx (3, 18, 24). These slime and glycocalyx together with host tissue proteins, and platelets form biofilm and fibrin

sleeve surrounding catheters. A biofilm is a highly organized structure. It embeds the microorganisms on the catheter surface and protects the microorganisms from attack by circulating polymorphonuclear neutrophils, antibiotics and other stress.

1.2.2 Urinary catheters

Microorganisms enter the urinary bladder in UCAI through either extraluminal route or intraluminal route of urinary catheters. Microorganisms traveling through the extraluminal route are mainly endogenous microorganisms from the rectum. These microorganisms migrate through the mucus film surrounding the external surface of catheters. Microorganisms traveling through the intraluminal route are usually exogenous, due to reflux of urine in contaminated urine bags (11).

1.3 Adhesion mechanisms

1.3.1 Definition of adhesion

Adhesion of microbial cells can be viewed as a population of originally free moving, randomly distributed microbial cells in air or in liquid concentrate on the interface between air or liquid and a surface (26). It is accompanied by a decrease in Brownian motion of microbial cells (27). In 1943, ZoBell proposed that adhesion of microbial cells could be described as a two-phase process. The first phase is a reversible step. Microbial cells are spontaneously attracted to a surface. Microbial cells on the surface are easily removed in this step. The second phase is a time

dependent irreversible step. Microbial cells adhere on a surface firmly and no Brownian motion is exhibited (27). This hypothesis was later confirmed by Marshall and associates. (27). Base on this observation, adhesion is defined by the time needed for achieving the irreversible step. The time needed has not been standardized. Some investigators defined adhesion as a process of microbial cells accumulating on a surface five minutes to two hours after addition of microbial cells (28).

1.3.2 Adhesion mechanism

Adhesion does not come naturally as the consequence of gravity. Rather, adhesion is the result of the interaction of different forces (29). Indeed, most surfaces of cells and of biological materials carry negative charges, thus two surfaces are repulsive in close proximity (29). Adhesion occurs because this repulsive force is counteracted and overcome by other attracting forces. This concept originates from the theory of Derjaguin, Landau, Verwey and Overbeek (DLVO theory) (30). In addition, molecular structures, such as pili of microbial cells also facilitate in breaking this repulsive force due to the small radii of curvature of these structures (29). The forces involved in the phase one and the phase two of adhesion will be described.

1.3.2.1 The phase one

Movement of microbial cells is mainly under the influence of flow field of surrounding fluid at the distance of more than 100nm from an adhering surface (31).

In addition, chemotaxis of microbial cells also contributed to the movement (32).

The forces contributed in the phase one of adhesion are physicochemical and non specific in nature (33). These forces can be further classified into long range interactions and short range interactions (33). The long range interactions refer to the forces involved at the distance of 20nm – 100nm between the cells and the adhering surface (31). Short range interactions refer to the forces involved at the distance of less than 20nm from the adhering surface (31). Mutual forces of van der Waals force, electrostatic forces, and hydrodynamic forces are involved in the long range interactions (31). Ionic bond, hydrogen bond, dipole interactions, and hydrophobic interactions are involved in the short range interactions (29). The repulsive force due to electric double layer between two adhering surfaces is also involved (29). Adhesion occurs when the interactions of these forces result in a decrease in the Gibb's free energy of the system (30). It is depicted by the equation of Extended DLVO theory:

$$\Delta G^{adh} = \Delta G^{vdW} + \Delta G^{dl} + \Delta G^{AB}$$

where G^{adh} is Gibb's free energy of adhesion, G^{vdW} is Gibb's free energy of van der Waals force, G^{dl} is Gibb's free energy of electric double layer interactions, G^{AB} is Gibb's free energy of acid-base interactions, which represents interactions such as hydration, and hydrophobic interactions.

The phase one of adhesion is influenced by factors changing the physical

properties of the two adhering surfaces as non specific physicochemical forces dominate in this phase. These factors include the hydrophobicity of two adhering surfaces (34), which in turn depending on the culture medium, surface charge of two adhering surfaces, temperature of the surrounding fluids, and concentration of electrolytes in the surrounding fluids (28). The list of the factors is not exhaustive.

1.3.2.2 The phase two

Molecular and cellular interactions between the molecular structures of two adhering surfaces contribute to the phase two of adhesion (33), resulting in a firm adhesion of microbial cells. The molecular structures involved are the adhesins in fimbriae, pili, capsule, and slime (33). The $F7_1$, F8, and F13 adhesins of fimbriated *Escherichia coli* (33), capsular polysaccharide adhesin PS/A, and slime associated antigen (SAA) of *Staphylococcus epidermidis* were found to mediate adhesion (30). Microbial cells secrete adhesive extracellular matrix to the surface (35). This is especially important to adhesion to inanimate surfaces. As interactions between ligands and receptors dominate in this phase, the factors affecting this phase include the density and accessibility of ligands and receptors (36).

1.4 Catheters

Different types of catheter are used for the access of arteries, veins, urinary system, and central nervous system. Indwelling urinary catheters and intravenous

catheters are the most commonly used catheters. Indwelling urinary catheters or Foley catheters are inserted into urinary bladder for the drainage of urine. The catheters usually are made of latex or silicone (34).

Intravenous catheters are further classified into PVC and CVC (33, 37). Peripheral intravenous catheters are usually inserted into metacarpal vein, dorsal venous arch, cephalic vein or basilic vein (38). The size of peripheral intravenous catheters ranges from 14 gauge to 22 gauge (38). These catheters are made of polyurethane, or in older times, polyvinyl chloride or Teflon®. Central venous catheters are further divided into peripherally inserted central catheters, tunneled central venous catheters, non tunneled central venous catheters, and implantable ports (33, 39). Central venous catheters are made of silicone or polyurethane (40).

Hydrophobicity is an important physical parameter of catheter materials. It influences the adhesion of microbial cells. Hydrophobicity is reflected by an water contact angle (41). The higher the value, the higher is the hydrophobicity. The water contact angle of FEP, polyurethane, and silicone is 78°, 108°, and 109° respectively (42).

1.5 Biology of *Candida albicans*

1.5.1 Taxonomy of *Candida albicans*

Candida albicans species is classified as phylum *Ascomycota*, class

Hemiascomycota, order *Saccharomyces*, family *Candidaceae*, and genus *Candida* (43). This classification scheme is based on the phylogentics study by comparing SSU rDNA sequences (44). *Candida albicans* species can be further classified into serotype A and serotype B according to the structure complexity of the mannan of cell wall mannoproteins (45, 46).

1.5.2 Morphology

Candida albicans is a dimorphic yeast because it undergoes reversible yeast-mycelium transition (47, 48, 49). However, it should be noted that its growth form is polymorphic (50, 51). It can grow in the forms of ovoid yeast cells, pseudohyphae, germ tubes, and hyphae. Yeast cells are unicellular and oval in shape with the size of about 3 x 5μm (47). It buds at the polar region of the cell. Pseudohyphae is a chain of the elongated yeast cells with constrictions at septa (49). Germ tube is the initial structure of the hyphae. It is a small elongated polar budding without constriction at septum. Hyphae is the filamentous growth form of *Candida albicans*. It arises from elongation instead of budding (48). Yeast cell formation is favored at temperature below 35℃ and at pH 6.5 or below (47, 52, 53). Germ tube formation takes place after 1-3 hours of incubation of yeast cells in serum at 37℃ (54). Germ tube formation is also induced by *N*-acetylglucosamine, and L-proline (55). Further incubation in serum results in hyphae formation (49). Hyphae formation

is also favored at pH 6.5, by deficiency of nitrogen and carbon sources and a microaerobic environment (53). It should be noted that formation of hyphae requires the combination of all the factors stated above (48).

1.5.3 Genome

The genome of *Candida albicans* is diploid (56) of 32Mbp (57). It is comprised of 16 chromosomes designated as R, 1-7 in the order of the largest chromosome to the smallest (57). *Candida albicans* is reproduced by mitosis. Ploidy changes due to loss of chromosomes in mitosis were observed (59, 60, 61, 62, 63). Only 60-70% of clinical isolates of *Candida albicans* possesses all of the 16 chromosomes (64).

Regions of intermediate repeat element called major repeat sequence (MRS) are present in the genome of *Candida albicans*. The MRS is comprised of a RPS region flanked by HOK and RB2 regions together. Their size is about 2 – 2.9kb, 8kb, and 6kb respectively. The MRS is present in all chromosomes except chromosome 3 (57, 58) although RB2 regions were found on chromosome 3. The size of these three elements, thus in turn, the size of the MRS varies from strain to strain (65, 66, 67). The MRS facilitates the translocation and deletion of chromosome fragments, resulting in genome variability and chromosome length polymorphism (68).

1.5.4 Biology of *Candida albicans* cell wall

1.5.4.1 Constituting molecules of *Candida albicans* cell wall

Candida albicans cell wall consists of β-glucan, chitin, proteins, mannoproteins, and lipids (69), accounting for 48 – 60%, 0.6 – 2.7%, 3 – 6%, 20 – 23%, and 2% of cell wall dry weight respectively (70). The composition of the β-glucans, and mannoproteins are similar in the cell wall of yeast cells, germ tubes, and hyphae cells. The chitin content is 4–5 folds higher in hyphae cells.

The β-1,3-glucan, and β-1,6-glucan are polymers of β-D-glucose molecules linked by β-1,3-glycosidic bond and β-1,6-glycosidic bond respectively. Chitin is an unbranched polymer of *N*-acetylglycosamine linked by β-1,4-glycosidic bond. Each β-1,3-glucan polymer consists of about 40–50 branches of β-1,3-glucan linked by β-1,6-glycosidic bond. One to two chains of β-1,6-glycan are branched from each β-1,3-glycan polymer through β-1,4-glycosidic bond. Chitin is linked to the β-1,3-glucan through β-1,4-glycosidic bond or associated by hydrogen bond (71). The two β-glucans, together with chitin provide the rigidity of the cell shape and the non-reducing ends for the linkage of cell wall proteins and lipids to the cell wall.

Mannoprotein is the protein linked with mannan by *N*- or *O*-glycosylation (69). Mannan is a polymer of mannose linked by α-1,6-glycosidic bond. *N*-glycosylated mannan links to the asparagine amino acid residues. *N*-glycosylated

mannan is highly branched and is classified into complex mannan structure, intermediate structure, and simple structure. Either a single mannose molecule or a short unbranched mannan polymer is linked to the serine or threonine amino acid residues of cell wall proteins in the *O*-glycosylated mannan.

1.5.4.2 Organization of *Candida albicans* cell wall

There are two models describing the organization of *Candida albicans* cell wall. *Candida albicans* cell wall is either viewed as a layered structure of at least 5 layers, depending on strains, growth forms, and culture media (47, 56, 72) or as a three dimensional structure of β-1,3-glucan polymers filled with proteins and lipids (73, 74, 75).

In the first model, *Candida albicans* cell wall consists of a layer of mannoproteins on the cell membrane, followed by a layer of β-glucan and chitin. The layer is stacked by a layer of β-glucan and another layer of mannoproteins. The outer layer contained appendages extending from the *Candida albicans* cell wall (47). These appendages are named as fimbriae. Each fimbria is made of mannoproteins with the size of about 66kDa, thus called MP66 (76, 77).

In the second model, polymers of β-1,3-glucan form a three dimensional network on the cell membrane of *Candida albicans*. β-1,6-glucan and chitin polymers are linked to the network through covalent bond or associated by hydrogen

bond. Cell wall proteins are either linked to the non reducing ends of β-1,3-glucan of the network directly or to the network via β-1,6-glucan by the glycosylphosphatidylinositol (GPI) anchor. Proteins linking to the β-1,3-glucan directly are called Pir-protein and the those linked through GPI anchor are called GPI-protiens. A group of GPI-proteins are called agglutinin-like sequence (Als) proteins, which possess adhesive function.

1.6 Agglutinin like sequence gene family

1.6.1 Gene structure of agglutinin like sequence genes

There are eight members in the agglutinin like sequence (*ALS*) gene family, namely *ALS1*, *ALS2*, *ALS3/ALS8*, *ALS4*, *ALS5*, *ALS6*, *ALS7*, and *ALS9*. It was discovered that *ALS3* and *ALS8* are the same gene. They localize on three of the eight chromosomes of *Candida albicans* (78). The *ALS1*, *ALS2*, *ALS4*, *ALS5*, and *ALS9* localize on the chromosome 6 (78, 79, 80, 81). The *ALS6* and the *ALS7* localize on chromosome 3 (82). The *ALS3* / *ALS8* localizes on chromosome R (83).

All *ALS* genes share a conserved three domains structure. All characterized *ALS* genes consist of a 5' domain, a tandem repeat domain of 108 base pair (bp), and a 3' domain (78). *ALS* genes exhibit high degree of inter-strain variability in the length. Reference to *Candida albicans* strain SC5314 (ATCC® number: MYA-2876), which is one of the most characterized strains (78), the length of different *ALS* genes ranges

from 3144 bp (*ALS3* smaller allele) to 6897 bp (*ALS7*) (82).

The length of the 5' domain ranges from 1299 bp to 1308 bp. The length of the tandem repeat is variable, depending on the number of tandem repeat unit of each *ALS* gene. The length of the 3' domain is also variable, ranging from 879bp (*ALS3*) to 4404bp (*ALS7*) (78). The 3' domain of *ALS7* is the longest among the other *ALS* genes (78) because another tandem repeat of 15 bp is embedded in the 3' domain of *ALS7* (82). The region was named as VASES region because the code for the amino acid sequence is VASES.

1.6.2 Sequence similarity

Each domain of the *ALS* genes shares a number of similar features although a high degree of inter allele and inter strain variability in the length of the genes is observed.

The length of the 5' domain of all *ALS* genes ranges from 1299 bp to 1308 bp (78), displaying little differences in length. The 5' domain shows 55% - 85% of identical sequence across the *ALS* gene family (78, 80, 82). The sequence of 5' domain of *ALS1*, *ALS3* / *ALS8*, and *ALS5* shares 85% of identity (81, 83). In addition, the 5' domains of all *ALS* genes possess no more than two *N*-glycosylation site coding sequences (78, 80, 82). The percentage of codons codes for serine and threonine, which represents possible *O*-glycosylation site, ranges from 25% - 31%.

The tandem repeat domains of *ALS1* to *ALS4* can cross hybridize to that of *ALS1* (80). The tandem repeat domains of *ALS5* to *ALS7* can cross hybridize to that of *ALS5* (82).

The 3' domains of all *ALS* genes have more *N*-glycosylation site coding sequences, and higher percentage of serine and threonine coding codons than the 5' domains of all *ALS* genes. It ranges from three to six sites, except none is found in the 3' domains of *ALS5* and of *ALS6* (80, 82). It ranges from 36% to 45%, compare to the 25% - 31% of the 5' domains (80, 82). A region coding for a GPI anchor addition site is found in the 3'domains of all *ALS* genes (78, 80, 81). The 3' domains of *ALS2* and *ALS4* share 95% of sequence identity (80), while that of *ALS6* and *ALS7* share 93% of sequence identity (82).

1.6.3 Sequence variability

Sequence variability between different alleles and different strains is contributed by differences in the number of tandem repeat present in different alleles and in the length of the 3' domains.

The number of tandem repeat unit contributes to most of the inter allele and inter strain differences of each *ALS* gene (78). For example, *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) is heterozygous for *ALS3* and *ALS5*. The larger *ALS3* allele has twelve repeating units and the smaller *ALS3* allele has nine

repeating units (84). *ALS1* exhibit inter strain differences in the number of tandem repeat unit. Three to seven of tandem repeat units were found in *Candida albicans* strain SB B311, V6, V6R, 1177, 3153A, CAI 4, and ATCC 18804 (79).

Sequence polymorphism is also observed. For example, there are 2.1% and 2.9% of sequence differences in the 5' domain and the 3' domain of *ALS5* between *Candida albicans* strain CAI and *Candida albicans* strain 1161 (81). Apart from sequence variability, some *ALS* genes are missing in some *Candida albicans* strains. For example, *Candida albicans* strains B311 and B792 do not have *ALS5* (81).

1.6.4 Expression of *ALS* genes

Expression of *ALS* genes is found to be associated with different physiological conditions. *ALS1* was first thought to be expressed in hyphae of *Candida albicans* (79). It was later found that expression of *ALS1* is induced by unknown substance(s), in RPMI 1640, and yeast peptone dextrose (YPD) broth (83). Expression of *ALS3* is hyphae specific. Expression of *ALS4* was detected in *Candida albicans* cells in mid-log phase (80). *Candida albicans* cells exhibit low level of *ALS5* expression in YPD medium.

Expression of different *ALS* genes was also detected *in vivo*. In an experiment conducted by Cheng and associates, all *ALS* genes were detected in the RNA extracted from clinical vaginal fluid specimens by reverse transcriptase

polymerase chain reaction (RT-PCR) (85). The percentage of detection of all *ALS* genes in patients with vaginal candidiasis symptoms was *ALS1*: 86%, *ALS2*: 71%, *ALS3*: 57%, *ALS4*: 21%, *ALS5*: 21%, *ALS6*: 14%, *ALS7*: 21%, and *ALS9*: 64% (85).

Inter strain variability in the expression level of *ALS* genes was observed. In an experiment conducted by Hoyer and associates, *ALS1* was not expressed in two *Candida albicans* strains grown in YPD medium (83). It was found that one (WO-1) of the two *Candida albicans* strains does not possess the promoter sequence of *ALS1* (83). The expression level of *ALS3* in RMPI 1640 of *Candida albicans* strains SC5314 and 3153A was higher than that of strain 1177, B311, B792, and WO-1 in another example (83).

1.6.5 The Als proteins

Als proteins localize evenly on the cell wall of *Candida albicans* cells. The proteins are linked to the cell wall of *Candida albicans* cells through β-1, 6-glucan by GPI anchor. The size of different non glycosylated Als proteins ranges from 117kDa to 245kDa, depending on the size of the *ALS* genes. However, as Als proteins are heavily glycosylated (78), the size of the glycosylated proteins is several times higher. For example, the size of non-glycosylated Als1 protein is 125kDa, and the size of the glycosylated Als1 protein is 600 kDa (78).

The N terminal domains of all Als proteins are relatively free of

glycosylation (78, 79, 80, 82, 83) and are hydrophobic. A secretory signal sequence of 17 bp is found at the start of the N terminal of all Als proteins (78, 79, 80, 82, 83). The sequences contribute to the localization of Als proteins on the cell wall. In an experiment conducted by Hoyer and Hecht, *Saccharomyces cerevisiae* transformed with the 5' domain of *ALS1* secreted N terminal domain proteins in culture medium. Deletion of the sequence resulted in the loss of secretion of the N terminal domain proteins (80). This demonstrated the importance of this sequence in the localization of the proteins. The N terminal is postulated to be an immunoglobulin (78, 81, 86). This postulation is supported by the high percentage of antiparallel β-sheet structure and the low percentage of α-helix structure in Als1 protein (86) and in Als5 protein (81). This structure pattern is exhibited in the immunoglobulin domain of other proteins (81). In addition, seven hypervariable regions were found in the N terminal domain of Als1 protein (86).

There is little information about the tandem repeat domain and the C terminal domain of Als proteins. It is known that both the tandem repeat domain and the C terminal domain are heavily *N*- and *O*-glycosylated. In addition, it is known that the C terminal domain of Als proteins is hydrophobic and there are two GPI anchor addition site coding sequences in the 3'domain (78, 80, 81).

1.6.6 Functions of Als proteins

It is postulated that Als proteins possess adhesive functions because of the similarity with the *α-agglutinin* of *Saccharomyces cerevisiae* (79), and the possible immunoglobulin like structure of the N terminal domain (78, 81, 86).

In an experiment conducted by Sheppard and associates, *ALS1*, *ALS3*, and *ALS5* transformed *Saccharomyces cerevisiae* adhered to gelatin, fibronectin, laminin, vascular endothelial cells, and FaDu epithelial cells (86). *ALS6* transformed *Saccharomyces cerevisiae* adhered to gelatin, and the *ALS9* transformed *Saccharomyces cerevisiae* adhered to laminin. *ALS7* transformed *Saccharomyces cerevisiae* showed no adherence to the substrata tested in that experiment. These proteins exhibited different substrate specificity. Although the *ALS1*, *ALS3*, and *ALS5* transformed *Saccharomyces cerevisiae* exhibited highest adherence to fibronectin, the adherence of *ALS3* clone to gelatin was about 50% lower than that of the *ALS1* and the *ALS5* clones. However, the adherence of *ALS1* and *ALS5* clones to FaDu epithelial cells was 50% lower than that of *ALS3* clone (86). In an experiment conducted by Gaur and Klotz, Als5 proteins adhered to fibronectin and laminin, which is consistent with the result of the experiment by Sheppard and associates, and to collagen IV (87). In another experiment conducted by Zhao and associates, Als2 protein and Als4 protein adhered to vascular endothelial cells (88). Als2 protein also adhered to reconstituted human oral epithelial cells (89). The substrate specificity is associated

with the N terminal domain (86).

The adherence of Als proteins is influenced by the number of the tandem repeat unit. In an experiment conducted by Oh and associates, it was found that the adherence of Als3 larger allele protein of *Candida albicans*, which possesses 12 tandem repeat units, had a higher adherence than the Als3 smaller allele protein, which possesses 9 tandem repeat units to human umbilical vein endothelial cells and pharyngeal epithelial cells (85).

In the same experiment, it was found that deletion of Als2 protein or Als4 protein resulted in a reduction in germ tube formation.

1.7 Adhesion assay

Adhesion can be studied using high accuracy quantitative methods, relative quantitative methods, and semi-quantitative methods (90). High accuracy quantitative methods measure the absolute adhesive energy between two adhering surfaces. In this project, a semi-quantitative method measurement was used. As a result, only semi-quantitative methods will be discussed.

Adhesion assay consists of an adhesion model and a quantitation system. An adhesion model mimics the adhesion process of microbial cells to other cells or inanimate objects *in vitro* with a washing process for removing non-adherent microbial cells from the adhering surface. The quantitation system is any method

measuring the number of microbial cells remained adherent after washing (28).

1.7.1 Adhesion model

There are two types of adhesion model, namely static model and dynamic model (90). The static model refers to any adhesion model which microbial cells in a static cell suspension are allowed to sediment on cells or a surface for a certain period of time before washing. The dynamic model refers to any adhesion model passing cell suspensions through devices such as a parallel plate chamber and a cylindrical channel at a known flow rate. Microbial cells were subjected to the shearing force due to the flow of cell suspensions (41).

The advantage of a static model is the ease to perform adhesion experiment, and no special device is needed. However, the gravitational force dominates in the phase one of adhesion process and shearing forces due to blood flow, administration of drugs in intravenous catheters cannot be mimicked. The advantage of a dynamic model is that the effect of flow can be studied and controlled (41). However, special devices are needed and experiments are more difficult to be performed.

Adherence of microbial cells is represented by the percentage of initial inoculum adhered on substratum surface. The percentage of initial inoculum is calculated by the formula:

$$\text{Percentage of initial inoculum} = \frac{\text{Number of microbial cells adhered}}{\text{Number of cells in initial inoculum}} \times 100\%$$

The use of percentage of initial inoculum as an indicator of adherence is to standardize the results with the number of cells in the initial inoculum since small variations in number of cells may exist between the inoculum of different sample or between different batches of experiments. The use of percentage to represent adherence can ensure any difference in adherence detected is due to the adhesion properties of the microbial cells tested, instead of the variations in the cell number.

1.7.2 Factors affecting static adhesion model

A number of factors have to be considered in performing static adhesion assay. The factors include the inoculum size of microbial cells, bathing fluid, and incubation time. The topology and chemical composition of catheters also affect the results of an adhesion assay when catheters are used as substrata (28). It should be noted that the factors stated above are not exhaustive.

The inoculum size of microbial cells affects the probability of a cell adhering on to an adhering surface (33, 36). As the number of cells increases, the accessibility of an adhering surface to a cell decreases, and the surface may be saturated with microbial cells (33, 36). The purpose of an adhesion assay has to be

considered in determining the inoculum size. It is recommended that 10^6 to 10^8 cells should be used in an adhesion assay studying events happened within five minutes to two hours after addition of microbial cells. For adhesion assay studying microbial cells proliferation after adhesion, 10 to 10^6 cells should be used (28).

Bathing fluid influences the result of an adhesion assay by two fold. First metal ions such as magnesium ions and calcium ions can promote microbial cells adhesion to adhering surfaces by bridging two negative adhering surfaces. Second, the surface hydrophobicity of microbial cell surfaces and catheter surfaces is also affected by electrolyte composition (91).

The incubation time of adhesion assay ranged from 5 minutes to 24 hours (28). It is suggested that the incubation time for microbial cells to sediment on an adhering surface depends on the aim of adhesion assay (28). In adhesion assays aimed at studying initial event of adhesion, the incubation time should be 5 minutes to 2 hours, while the incubation time should be 6 hours to 24 hours for adhesion assays focus on cell proliferation after adhesion. However, it should be noted that there is no absolute standard on the incubation time to be used in adhesion assays.

Adherence of a strain of microbial cells in relative measurement is represented by the percentage of initial inoculum adherent on an adhering surface. As a result, the number of cells adherent on an adhering surface is a function of the

surface area (33, 34). Minor differences in the microtopology or smoothness, and manipulations on adhering surfaces such as cutting of catheters introduce variations in catheter surface area. Additives are added in catheters materials during production of catheters (33, 34). The chemical nature and composition of these additives are usually commercial secrets and vary between manufacturers (34). These additives also introduce variations, which can hardly be controlled.

1.7.3 Quantitation methods of adherent cells

Quantitation methods of adherent cells on catheter surfaces include sonication and culture of detached cells, staining of adherent cells, ATP bioluminescence, direct observation using optical microscope or scanning electron microscope, enzyme linked immunosorbent assay (ELISA), radiolabeling, and counting of detached cells (28, 90). It should be noted that the above methods are not exhaustive. Only the first three methods will be introduced.

1.7.3.1 Sonication

Adherent cells on catheter surfaces are removed by sonicating and vortexing catheters submerged in PBS in sterile test tubes or bijou bottles. The number of cells in PBS is then determined by viable count. The advantage is that number of adherent cells on catheters of different length and shape can be measured by this method. However, adhered cells on catheter surfaces may remain on catheter

surfaces after sonication. In addition, sonication may also lead to cell disruption (28). Both lead to underestimation of count. Preliminary experiments for determining the optimal power and oscillation frequency is favorable.

1.7.3.2 Staining methods

Adherent cells on catheters are stained using safranin or trypan blue (92). The adherent cells are destained using sodium dodecyl sulfate (SDS) buffer. The absorbance of the SDS buffer is measured. The number of cells is determined using a standard curve of absorbance against number of cells. This method is easy and inexpensive (92). This method may over estimated the number of adherent cells as slime and extracellular matrix, which is not directly proportional to the number of cells, are also stained by safranin or trypan blue.

1.7.3.3 ATP bioluminescence

Catheter sections or fragments are placed in bioluminescence reagents for the extraction of ATP from cells and for bioluminescence reaction. Bioluminescence is measured. The number of cells is determined using standard curve of bioluminescence against number of cells. This method is more sensitive than staining method (28). However, it may over estimate the number of adherent cells as ATP from dead cells is extracted for bioluminescence reaction also. A standard curve should be prepared for each strain of microbial cells tested as the number of ATP molecules per

cell vary from strain to strain.

1.8 Research model

In this study, the adhesion properties of the *ALS* gene were studied using a *Saccharomyces cerevisiae* model. The *ALS* genes of *Candida albicans* were transformed into *Saccharomyces cerevisiae* and the adherence of the transformed *Saccharomyces cerevisiae* was measured. In effect, the functions of the *ALS* genes of *Candida albicans* were reflected by the functions of the *ALS* genes transformed into *Saccharomyces cerevisiae*. The results obtained in this study would only be valid if the Als proteins translated in *Saccharomyces cerevisiae* retain their native structures and functions as in *Candida albicans* cells. As in the use of other model organisms, this research model is based on the assumption that *Candida albicans* and *Saccharomyces cerevisiae* are the descents of a common ancestor and their metabolic, developmental pathways and genetics materials are largely conserved. As a result, the Als proteins translated in *Saccharomyces cerevisiae* should retain their native structures and functions. This is supported by the fact that *Candida albicans* and *Saccharomyces cerevisiae* belongs to the same order *Saccharomyces* and are close relatives (93). In addition, there are evidences suggesting that *Candida albicans* and *Saccharomyces cerevisiae* are largely conserved at the level of transcription (94) and the biosynthesis of GPI proteins (95), which Als protein is one of them.

In addition, the phenotypic effect of the model organism after genetic manipulations should be easily observed. In adhesion study, the adherence of the transformed *ALS* genes (or other genes) should not be masked by the basal adherence of the *Saccharomyces cerevisiae* itself. It was known that *Saccharomyces cerevisiae* possesses low level of adherence to a number of substrata such as fibronectin, human epithelial cells, and polystyrene (86, 96, 97). A number of adhesive genes were found in *Saccharomyces cerevisiae* including *FLO1*, *FLO5*, *FLO9*, *FLO10*, *FLO11*, *FIG2*, and *α-agglutinin* (98, 99, 100). However, only *FLO11* conferred *Saccharomyces cerevisiae* adherence to agar and plastics (98), while the others were responsible for the flocculation and mating of *Saccharomyces cerevisiae* cells, instead of conferring adherence to other substrata (98, 99, 100) while a number of adhesive genes were found in *Candida albicans* including *HWP1*, *EPA1*, and different *ALS* genes (101). The low adherence of *Saccharomyces cerevisiae* to a number of substrata prevents the adherence of Als proteins from being masked.

Chapter 2 Aim of study

The aim of this study is (i) to study the effect of inoculum size in an adhesion model of catheters and to establish a quantitation system for the adhesion model; and (ii) to study the effect of transformation of *ALS1, ALS5* smaller allele, and *ALS6* to *Saccharomyces cerevisiae* on its adhesion to human fibroblasts, and to tetrafluoroethylene and hexafloropropylene polymer (FEP), polyethylene, and silicone catheters.

The effect of transformation of *ALS1*, *ALS5* smaller allele, and *ALS6* to *Saccharomyces cerevisiae* on its adherence to human fibroblasts and to FEP, polyurethane, and silicone catheters has never been reported. The study can provide additional information on the function of the three genes in adhesion.

Chapter 3 Materials and Methods

3.1 Preparation of bacteriological reagents

Phosphate buffered saline was prepared by dissolving 10 phosphate buffered saline (Dulbecco A) tablets (Oxoid, UK) in 1L of double distilled water. The buffer was then autoclaved at 121℃, 103kPa for 15 minutes. Phosphate buffered saline was used for washing process in the culture of fibroblasts, *Candida albicans* cells, *Saccharomyces cerevisiae* cells and for the washing process in adhesion assays.

Sabouraud dextrose agar (SDA), YPD agar and YPD broth were prepared for the culture of *Candida albicans* and of *Saccharomyces cerevisiae*. Sabouraud dextrose agar was prepared by dissolving 65g of sabouraud dextrose agar powder (Oxoid, UK) in 1L of double distilled water. The agar was then autoclaved at 121℃, 103kPa for 15 minutes. Yeast peptone dextrose agar was prepared by dissolving 65g of yeast peptone dextrose agar powder (Sigma, USA) in 1L of double distilled water. The YPD agar was then autoclaved at 121℃, 103kPa for 15 minutes. Yeast peptone dextrose broth was prepared by dissolving 50g of yeast peptone dextrose broth powder (Sigma, USA) in 1L of double distilled water. The dissolved powder was then autoclaved at 121℃, 103kPa for 15 minutes.

Cornmeal tween 80 agar and chromagar were prepared for confirmation of identity of *Candida albicans* and of *Saccharomyces cerevisiae*. Cornmeal tween 80

agar was prepared by dissolving 17g of cornmeal agar powder (Oxoid, UK) in 1L of double distilled water. Ten milliliter of tween 80 (Sigma, USA) was added. The agar was boiled to dissolve. The cornmeal tween 80 agar was then autoclaved at 121℃, 103kPa for 15 minutes. Chromagar was prepared by dissolving 47.7g of CHROMagar™ Candida (CHROMagar, France) in 1L of double distilled water. The dissolved agar was autoclaved at 121℃, 103kPa for 15 minutes.

Horse serum for germ tube test of *Candida albicans* and of *Saccharomyces cerevisiae* was purchased from Oxoid, UK. Biochemical tests of *Candida albicans* and of *Saccharomyces cerevisiae* were performed using API 20C Aux (Biomerieux, France).

3.2 Confirmation of identity of *Candida albicans* and of *Saccharomyces cerevisiae*

Identity of presumptive *Candida albicans* and of *Saccharomyces cerevisiae* was investigated by the morphology test on cornmeal agar, germ tube formation in horse serum, biochemical tests, and colony color on chromagar. Presumptive *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) and presumptive *Saccharomyces cerevisiae* (ATCC® number: 96686) were cultured on SDA at 30℃ for 48 hours. Isolated colonies on the SDA were used for the four tests stated above.

Morphology test on cornmeal agar was performed by incubating presumptive *Candida albicans* and presumptive *Saccharomyces cerevisiae* on cornmeal agar. The presence of the structure of hyphae, pseudohyphae, chlamydospores, and blastoconidia on the agar was used for the identification of *Candida albicans* and of *Saccharomyces cerevisiae*. Morphology test on cornmeal agar was performed by making a 1cm cut on the cornmeal agar surface. Then a single isolated colony of *Candida albicans* or of *Saccharomyces cerevisiae* was streaked across the cut perpendicularly. A flamed cover slip was placed over the inoculated region. The cornmeal agar was incubated at 30℃ for 48 hours. Morphology of the *Candida albicans* and of *Saccharomyces cerevisiae* was observed using a light microscope. Clusters of blastoconidia located around the septa of hyphae, and single round chlamydospore at the terminal of hyphae should be observed for a *Candida albicans* culture (102). Large yeast cells of various shapes, absence of hyphae should be observed for a *Saccharomyces cerevisiae* culture (102). Scanty amount of short pseudohyphae may be observed.

Germ tube test was performed by inoculating of presumptive *Candida albicans* and presumptive *Saccharomyces cerevisiae* in horse serum and incubated at 37℃ for 1 hour to 3 hours, followed by observation of germ tube formation under light microscope. Germ tube without constriction at the point of origin should be

observed for a *Candida albicans* culture. Germ tube–like structure with a constriction at the point of origin should be observed for a *Candida tropicalis* culture (102). Other microorganisms showing no germ tube should be considered as non *Candida albicans*.

Biochemical tests (carbohydrate assimilation tests) of presumptive *Candida albicans* and of *Saccharomyces cerevisiae* were performed using commercially available packaged strips API 20C Aux (Biomerieux, France). The ability to assimilate D-glucose, glycerol, calcium 2-keto-gluconate, L-arabinose, D-xylose, adonitol, xylitol, D-galactose, inositol, D-sorbitol, methyl-αD-glucopyranoside, *N*-acetyl-glucosamine, D-cellobiose, D-lactose, D-maltose, D-saccharose, D-trehalose, D-melezitose, and D-rafinose were tested. The assimilation pattern of a culture is represented by a profile index (97). The profile index is compared with the profile database of the company, giving the probability of different possible identities of the culture.

Biochemical tests were performed by resuspending a single isolated colony of presumptive *Candida albicans* and presumptive *Saccharomyces cervisiae* in the suspension medium provided from the kit to 0.5 Mcfarland. The inoculum was added into the wells of two API testing strips. The strips with the culture chambers were incubated at 30℃ for 72 hours. The turbidity of each well was recorded and the

profile index of the two cultures was calculated. The profile index was compare with the database.

Colony color on chromagar was performed for identifying *Candida albicans*. Presumptive *Candida albicans* was cultured on a commercially available chromogenic agar. Metabolic enzymes of different yeasts metabolize the chromgenic substrates in the agar into products of different colors. The colony color of a *Candida albicans* culture should be green. The test was performed by streaking an isolated colony of the presumptive *Candida albicans* on a chromagar. The chromagar was incubated at 30℃ for 48 hours. The color of the colonies was recorded.

3.3 Cell culture of fibroblasts

3.3.1 Preparation of cell culture reagents

Minimum essential medium was prepared for the culture of fibroblasts. Trypsin-ethylenediaminetetraacetic acid (EDTA) was prepared for the trypsinisation of fibroblasts in subculture.

Minimum essential medium was prepared by mixing 50mL of MEM 10X concentrate, 5mL of 200mM glutamine, 10mL of 1M hepes, 6mL of 7.5% sodium bicarbonate, 3mL of 4mg/mL gentamycin, and 50mL of bovine serum albumin with 400mL of autoclaved double distilled water. All the cell culture reagents and chemicals were from Gibco, USA. Trypsin-EDTA for trypsinisation of fibroblasts was

prepared by mixing 1.25mL of 2% sodium-EDTA (Sigma, USA) with 2.5% trypsin solution (Sigma, USA). All the reagents were prepared in a class II biosafety cabinet.

3.3.2 Recovery of freezing fibroblasts

Fibroblasts (ATCC® number: CCL-171) was a deposit from the Department of Microbiology, the Chinese University of Hong Kong. The frozen stock was at the stage of 22nd passage. It was kept in the cryovial with 1mL of minimum essential medium (MEM) (Gibco, USA) and supplemented with 5% of bovine serum albumin (Gibco, USA) and 5% of dimethyl sulfoxide (Sigma, USA). The cryovial was stored in liquid nitrogen.

One milliliter of fibroblasts (ATCC® number: CCL-171) deposited in liquid nitrogen was defrosted at 37℃ using a water bath. The fibroblasts were transferred into a 15mL centrifuge tube containing 5mL of MEM. The centrifuge tube was centrifuged at 5600rcf (relative centrifugal force) for 5 minutes at room temperature. The supernatant was removed. The pellet was resuspended in 5mL of MEM in a T_{25} flask. The T_{25} flask was incubated at 37℃ in 5% CO_2 and 95% relative humidity (RH).

3.3.3 Establishment of cell line

The confluence of fibroblasts was checked after 72 hours. The fibroblasts would be further incubated for 24 hours if the confluence of fibroblasts was less than

90%. The fibroblasts would be subcultured to a T_{75} flask if attaining 90% of confluence. The MEM in the T_{25} flask was removed. The fibroblasts were washed twice with 5mL of PBS. Two milliliter of trypsin-EDTA was added into the flask and incubated at 37℃ for 5 minutes. Complete dislodgement of fibroblasts was checked under an Eclipse TE 2000-S phase contrast microscope (Nikcon, USA). The fibroblasts were considered to be completely dislodged if all the fibroblasts were rounding off. The flask was slightly tapped if the fibroblasts were not completely dislodged. Three milliliter of MEM was added into the flask for stopping the action of trypsin. The cell suspension was centrifuged at 5600rcf for 5 minutes. The supernatant was removed. The pellet was resuspended in 10mL of MEM. The fibroblasts were incubated in a T_{75} flask at 37℃ in 5% CO_2 and 95% RH. The fibroblasts were passed to another T_{75} flask every two days.

3.4 Preliminary study of adherence of *Candida albicans* to fibroblasts and to catheters

3.4.1 Adherence to fibroblasts

3.4.1.1 Preparation of fibroblasts

The confluence of fibroblasts in a T_{75} flask was observed 48 hours after seeding. Fibroblasts would be further incubated for 24 hours if confluence was less than 80%. Fibroblasts would be harvested if 80% of confluence was attained. The

number of fibroblasts harvested was counted using a Fuchs-Rosenthal counting chamber. After counting, 2 x 10^6 of fibroblasts were seeded in each well of two 6-well cell culture plates. The fibroblasts were cultured at 37℃ in 5% CO_2 and 95% RH for 24 hours. The confluence of the fibroblasts was checked under a phase contrast microscope. The fibroblasts were further incubated for 24 hours if 100% confluence was not attained. The fibroblasts were used for adhesion assay of *Candida albicans* cells if 100% confluence of fibroblasts was attained.

3.4.1.2 Preparation of culture of *Candida albicans* and of *Saccharomyces cerevisiae*

Candida albicans strain SC5314 (ATCC® number: MYA-2876) and *Saccharomyces cerevisiae* (ATCC® number: 96686) were cultured on two YPD agar at 30℃ for 48 hours. Single isolated colony of *Candida albicans* and of *Saccharomyces cerevisiae* was subcultured in two flasks of 30mL of YPD broth at 30℃ for 18 hours. Afterwards, the growth of the *Candida albicans* and of the *Saccharomyces cerevisiae* was measured by absorbance at 600nm. The optical density (OD) of two flasks of 100mL fresh YPD broth was adjusted to 0.4 by adding an appropriate volume of the overnight *Candida albicans* cell suspension or *Saccharomyces cerevisiae* cell suspension. The volume of the two overnight cell suspensions added was calculated according to the following formula:

$$\text{Volume of cell suspension needed} = \frac{0.4 \times 100\text{mL}}{OD_{600nm} \text{ of overnight cell suspension}}$$

The absorbance of the two flasks of fresh YPD broth was measured after the addition of the *Candida albicans* cell suspension or the *Saccharomyces cerevisiae* cell suspension for confirmation before incubation. The flasks were incubated at 30℃ for 3 hours. Afterwards, the absorbance of the two flasks of fresh YPD broth was measured. The *Candida albicans* cells and *Saccharomyces cerevisiae* cells in the two flasks of fresh YPD broth were considered to be in mid-log phase if the absorbance at 600nm was between 0.8 and 1.0. The flasks were further incubated for 1 hour if the absorbance at 600nm was less than 0.8. The *Candida albicans* cells and the *Saccharomyces cerevisiae* cells in mid-log phase were centrifuged at 3528rcf for 5 minutes. The supernatant was discarded. The pellets were washed twice with 5mL of PBS and then resuspended in 5mL of YPD broth. The two cell suspensions were placed on ice for maintaining *Candida albicans* cells and *Saccharomyces cerevisiae* cells in mid-log phase until the subsequent counting of *Candida albicans* and *Saccharomyces cerevisiae* cells was finished. The number of *Candida albicans* cells and of *Saccharomyces cerevisiae* cells in the YPD broth was measured using a Fuchs-Rosenthal counting chamber. The two cell suspensions were diluted with YPD broth to 300cell/mL. The cell concentration of the *Candida albicans* cell suspension

and that of *Saccharomyces cerevisiae* cell suspension was confirmed by spreading 200μl of cell suspension on each of three SDA for each cell. The agar plates were incubated at 30℃ for 48 hours. The number of colony forming unit (CFU) was counted. The *Candida albicans* cell suspension and the *Saccharomyces cerevisiae* cell suspension were used immediately for the subsequent adhesion assay.

3.4.1.3 Adhesion assay

One milliliter of the *Candida albicans* cell suspension was added to each of the three wells of a 6-well cell culture plate seeded with fibroblasts. One milliliter of the *Saccharomyces cerevisiae* cell suspension was added to each of the three wells of the 6-well cell culture plate. One milliliter of YPD broth was added to 3 control wells of another 6-well plate instead of adding cell suspension. The two cell culture plates were incubated at 37℃ in 5% CO_2 for 30 minutes. Afterwards, the cell suspensions and the broth were removed. The fibroblasts were washed with 1mL of PBS twice. Six hundred microliter of trypsin- EDTA was added into each well for trypsinisation of the fibroblasts. The plates were incubated at 37℃ in 5% CO_2 and 95% RH for 5 minutes. Two hundred microliter of the trypsin- EDTA was plated on each of 2 SDA (1 well 3 SDA). The residual trypsin- EDTA was plated on the last SDA. The SDA were incubated at 30℃ for 48 hours. The CFU of each SDA was counted. The number of *Candida albicans* cells adhered on 1 well of fibroblasts was

the sum of the CFU of the 3 SDA of the well. The percentage of initial inoculum adhered on fibroblasts was calculated by the following formula:

$$\text{Percentage of initial inoculum} = \frac{\text{CFU adhered on fibroblasts}}{\text{CFU of initial inoculum}} \times 100\%$$

Two independent experiments were conducted on two different days for confirming the results.

3.4.2 Adherence to catheters

3.4.2.1 Preparation of catheters

Tetrafluoroethylene and hexafloropropylene polymer catheters were the 18 G x 1.88 inch BD Angiocath™ peripheral venous catheter (1.3mm x 48mm) (Becton Dickinson, USA). Polyurethane catheters were the Leaderflex (Central venous catheter made of polyurethane. Catalogue number: 121.08, Vygon, France). Silicone catheters were the Lifecath (Silicone Central Venous Catheter with Dacron Cuff for Long Term Tunnelled Use. Catalogue number: 2193.96, Vygon, France). Twelve 1cm FEP catheters fragments, twelve 1cm polyurethane catheter fragments and six 1cm silicone catheter fragments were prepared using a pair of sterile scissors, ruler and a pair of sterile forceps in a class II biosafety cabinet.

3.4.2.2 Adhesion assay

The catheter fragments were put into 30 sterile 5mL screw-capped glass bijou bottles, one catheter fragment per bijou bottle. *Candida albicans* cell suspension was prepared as described in Section 3.4.1.2 except that the *Candida albicans* cell suspension was diluted to $1x10^5$cell/mL instead of 300cell/mL. One milliliter of the *Candida albicans* cell suspension was added into 6 bijou bottles with FEP catheter fragments, 6 bijou bottles with polyurethane catheter fragments and 3 bijou bottles with silicone catheter fragments. One milliliter of YPD broth was added in each of the remaining bijou bottles as control. The bijou bottles were incubated at 37℃ in 5% CO_2 with the caps loosened for 30 minutes. Afterwards, the catheter fragments were transferred aseptically into 30 new sterile 5mL screw capped glass bijou bottles, one catheter fragment per bijou bottle. The catheter fragments were washed with 1mL of PBS twice. Three of each type of catheter fragments incubated in *Candida albicans* cells suspensions and three of each type of catheter fragments incubated in YPD broth were transferred into 18 new sterile 5mL screw capped glass bijou bottles, one catheter fragment per bijou bottle. Three milliliter of YPD broth was added to each of the 18 bijou bottles. The bijou bottles with catheter fragments and the control bijou bottles were incubated at 30℃ for 24 hours. The turbidity of the YPD broth was compared visually. *Candida albicans* cells were considered to be adhering on the catheter fragments if the YPD broth was turbid. *Candida albicans* cells were

considered to be not adhering on the catheter fragments if the YPD broth was clear.

For the remaining catheter fragments, one milliliter of methanol was added into each bijou bottle for 2 minutes at room temperature. Then the methanol was aspirated. The catheter fragments were air-dried at room temperature for 5 minutes by removing the caps of bijou bottles. The bijou bottles were covered by filter paper for preventing the catheter fragments from contamination of microorganisms from environment. One milliliter of acridine orange was added to each bijou bottle for 2 minutes. Acridine orange stain was prepared by dissolving 20mg of acridine orange powder (Sigma, USA) in 190mL of 1M sodium acetate solution (98). Then the acridine orange was aspirated. The catheter fragments were washed with 1mL of PBS twice and air dried as described above. Catheter fragments surface were observed for the presence of orange spots using a Leitz Laborlux 12 phase contrast darkfield microscope (Leica, Germany). Presence of orange spots on catheter surface was considered to be adhered *Candida albicans* cells. Silicone catheter was not tested for acridine orange staining because silicone catheter was opaque. The adherence of *Candida albicans* cells to silicone catheter fragments was tested by the turbidity test only.

3.5 Confirmation of expression of *ALS1*, *ALS5* smaller allele, and *ALS6* of *Candida albicans* in YPD broth

3.5.1 RNA extraction of *Candida albicans*

Culture of *Candida albicans* strain SC5314 (ATCC® number MYA-2876) was prepared as described in Section 3.4.1.2 except that the cell suspension was diluted with YPD broth to $1x10^7$cell/mL and *Saccharomyces cerevisiae* was not cultured. One milliliter of *Candida albicans* culture in YPD broth was centrifuged at 1209rcf for 5 minutes. The pellet was washed with 1mL of PBS twice. The pellet was resuspended in 100µl of Buffer Y1 in a 1.5mL microcentrifuge tube for digesting the cell wall. Buffer Y1 was prepared by dissolving 18.22g of sorbital (Sigma, USA) and 2.92g of EDTA (Pharmacia, USA) in 100mL of autoclaved double distilled water. Lyticase (Sigma, USA) at the concentration of 50U per $1x10^7$ *Candida albicans* cells and 0.1% of β-mercaptoethanol (Merck, Germany) were added into Buffer Y1 just before use. The tube was incubated at 30℃ using a water bath for 30 minutes. RNA was extracted after lysis of cell wall using RNeasy Mini Kit (Qiagen, USA) according to the instructions of manufacturer. Briefly, 350µl of Buffer RLT was added for the lysis of the spheroplasts. The tube was vortex at the top speed of a Maxi Mix II Vortex Mixer (Thermolyne, USA) for 10 seconds. Then 250µl of 96% ethanol (Beijing Reagent, China) was added. The solution was transferred to a spin column provided. The column was centrifuged at 22,713rcf for 15 seconds using the same centrifuge. Then 700µl of Buffer RW1 was added to the spin column. The column was

centrifuged at 22,713rcf for 15 seconds. Then 500µl of Buffer RPE was added to the column. The column was centrifuged at 22,713rcf for 15 seconds. Then 500µl of Buffer RPE was added to the column. The column was centrifuged at 22,713rcf for 2 minutes using the same centrifuge. The RNA was eluted in 30µl of Ultrapure distilled water at 72℃ (Gibco, USA). The RNA was stored at -80℃.

3.5.2 RT-PCR of *ALS1, ALS5* smaller allele, and *ALS6*

3.5.2.1 Primers

Primers were designed using software GeneTool™ (BioTools, Canada). The primers were designed according to the following criteria. First, the forward and reverse primers of each gene started from the two termini of the corresponding gene. Second, the length of the primers was not higher than 25 bases apart from the sequences of restriction digestion sites added and the capping sequences ahead of the restriction sites. Restriction digestion sites were added because the primers designed would be used in the polymerase chain reaction (PCR) of the three *ALS* genes. Third, the difference in the melting temperature (T_M) of the two primers of each gene was not higher than 5℃. Fourth, no false priming to other region of the gene was exhibited. Fifth, no significant alignment with other genes of *Candida albicans* was exhibited. The alignment of the primers with other genes of *Candida albicans* was performed using nucleotide-nucleotide blast program (blastn) of National Center for

Biotechnology Information (NCBI). The accession number of *ALS1* of *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) was XM_712984 XM_436303, *ALS5* smaller allele sequence was AY227439, and *ALS6* sequence was AY225310.

The *ALS1* was amplified using forward primer 5'-TAGGGCGGATCCCATGCTTCAACAATTTACATT-3' and reverse primer 5'-TAGGGCGCGGCCGCTCACTAAATGAACAAGGACA-3'. *ALS5* smaller allele was amplified using forward primer of *ALS1* and reverse primer 5'-TAGGGCCGGCCGTCATAGAAAGAAGAATAA-3'. *ALS6* was amplified using forward primer 5'-TAGGGCCGGCCGATGAAGACAGTAATACTATT-3' and reverse primer 5'-TAGGGCTCTAGATCATAAGAAGAAGAATAAT-3'. All the primers were purchased from Invitrogen, USA. Each of the primer powders was dissolved in Ultrapure Distilled Water (Gibco, USA) to the concentration of 100μM. Five microliter of forward primer and 5μl of reverse primer were mixed in 90μl of Ultrapure Distilled Water (Gibco, USA) as working stock (10μM).

3.5.2.2 RT-PCR

Each RT-PCR reaction consisted of 10μl of 2X buffer of SuperScript III One Step RT-PCR Platinum *Taq* high fidelity (Invitrogen, USA), containing 3.2mM of magnesium chloride and 400μM of dNTP, 1μl of 10μM primers, 1μl of enzyme mix, 0.5μl of RNA extracted from *Candida albicans* strain SC5314 (ATCC® number:

MYA-2876), and each reaction was made up to 20µl by Ultrapure Distilled water (Gibco, USA). Tetrad thermocycler (MJ research, USA) was preheated to 50℃ before the start of RT-PCR. Reverse transcription of the three *ALS* genes were conducted at 50℃ for 30 minutes. The reactions were heated to 94℃ for 2 minutes for inactivation of reverse transcriptase and for the activation of *Taq* polymerase. Thirty five cycles of PCR were performed using the following cycling parameters: 94℃ of denaturation for 10 seconds, 56℃ of annealing for 30 seconds and 68℃ of extension for 5 minutes. The RT-PCR was completed after a final extension at 68℃ for 15 minutes.

The RT-PCR product of each gene was mixed with 4µl of loading dye and was loaded in a 1.5% agarose gel. The agarose gel was prepared by dissolving 3g of Agarose for separation > 500bp. Ultrapure, MB Grade (USB, USA) in 200mL of TBE buffer of 0.5X strength. Tris-borate EDTA buffer was prepared by dissolving 33.73g of Tris-borate EDTA (USB, USA) in 200mL of double distilled water to formulate a 10X concentrate. Two hundred milliliter of the 10X concentrate was added into 3800mL of double distilled water to formulate a 0.5X buffer. The agarose was boiled using a household microwave until completely dissolved. The gel was poured into a gel tray loaded with a gel comb. The length of each well was 0.5cm, the width was 0.2cm, and the depth was 1cm. The gel was immersed in TBE buffer with 0.5X strength in a gel running tank. One microliter of 1kb trackit molecular marker

(Invitrogen, USA) was loaded into one of the well. Gel electrophoresis was performed at 120V for 1 hour. The agarose gel was stained with ethidium bromide for 30 minutes. The gel was observed under ultraviolet light. Photo record of the gel was taken.

3.6 Establishment of quantitation system of adhesion assay

Two detection methods for adhesion assay of *Candida albicans* cells and of *Saccharomyces cerevisiae* to catheters were tested in order to measure the number of cells adhered on catheters. The first method was the measurement of the absorbance of *Candida albicans* cells stained with safranin. Another method was the measurement of ATP bioluminescence of *Candida albicans* cells. Detection method having a broarder linear range and a lower detection limit was considered to be a more sensitive method and was adopted.

3.6.1 Absorbance measurement of *Candida albicans* stained with safranin

3.6.1.1 Preparation of *Candida albicans* culture

Candida albicans strain SC5314 (ATCC® number: MYA-2876) culture was prepared as described in Section 3.4.1.2 except that the pellet was resuspended in 5mL of RPMI medium 1640 (1X), Liquid, with L-glutamine (Gibco, USA) instead of 5mL of YPD broth. *Saccharomyces cerevisiae* was not cultured. The number of *Candida albicans* cells was counted using a Fuchs-Rosenthal counting chamber. The *Candida albicans* cell suspension was diluted with RPMI 1640 (Gibco, USA) to

$1x10^8$cell/mL. One hundred microliter of the cell suspension was serially diluted with 900µl of RPMI 1640 (Gibco, USA) by every ten fold to 1x10cell/mL for the establishment of standard curve.

Cell concentration of the cell suspensions prepared was confirmed by viable count. One hundred microliter of cell suspensions of cell concentration $1x10^8$cell/mL, $1x10^7$cell/mL, $1x10^6$cell/mL, $1x10^5$cell/mL and $1x10^4$cell/mL was serially diluted with 900µl of PBS for 5, 4, 3, 2 and 1 times respectively. One hundred microliter of the PBS diluted cell suspensions, and cell suspensions at the concentration of $1x10^3$cell/mL, $1x10^2$cell/mL, and 1x10cell/mL were spread on each of 3 SDA. The SDA were incubated at 30℃ for 48 hours. The number of CFU on the agar was counted. The number of cells in cell suspensions at each cell concentration was calculated by the formula:

Number of cells per milliliter = Number of CFU x Dilution Fold x 10

The number of CFU was multiplied by the factor of ten because only 100µl of cell suspension was spread on SDA.

3.6.1.2 Staining of *Candida albicans*

Four milliliter of the cell suspensions of different cell concentration was

added to each of the 3 wells of five 6-well cell culture plates (Becton Dickinson, USA). The plates were incubated at 37℃ in 5% CO_2 and 95% RH for 1 hour. Afterwards, the cell suspension in each well was removed to 27 sterile 5mL screw capped glass bijou bottles for subsequent viable count, 1 well per bottle. Each well was washed with 1mL of PBS twice. The PBS was removed to another 54 sterile 5mL screw capped glass bijou bottles for subsequent viable count, 1 well per bottle. One milliliter of safranin was added to each well for 15 minutes at room temperature. Safranin stain was prepared by dissolving 0.1g of safranin powder (Sigma, USA) in 100mL of autoclaved double distilled water for staining the *Candida albicans* cells. The safranin was removed. The wells were washed with 1mL of PBS twice. One milliliter of SDS buffer was added into each well for 30 minutes for destaining. Sodium dodecyl sulfate buffer was prepared by mixing 0.1g of sodium dodecyl sulfate (Sigma, USA) in 100mL of autoclaved double distilled water. Two hundred microliter of these SDS buffer from each well was added into each of the 3 wells of a 96-well microtiter plate. The absorbance of the SDS buffer at 490nm was measured by OpsysMR 96-well microplate reader (Dynex, USA).

3.6.1.3 Viable count of *Candida albicans* adhered on the 6-well plate

The cell suspensions were transferred to bijou bottles after adhesion, and the PBS transferred to bijou bottles after washing were cultured on SDA for the

calculation of *Candida albicans* cells adhered on the cell culture plates. Three SDA were used for each removed cell suspension and each removed PBS in bijou bottles. One hundred microliter of the removed cell suspensions from each bijou bottle, and 200μl of the removed PBS from each bijou bottle was spread on each of 3 SDA. The SDA were incubated at 30℃ for 48 hours. The number of CFU of each SDA was counted. The total number of cells present in cell suspensions after adhesion and in the PBS after washing was calculated proportionally. The number of cells adhered on the 6-well plate was determined by the following formula:

$$\text{Number of cells adhered} = \text{number of cells in cell suspensions after adhesion} - \text{number of cells in PBS after washing}$$

A graph of absorbance at 490nm against the number of cells adhered on 6-well plate was plotted for determination of the standard curve of this method. Two independent experiments were conducted on two different days for confirming the results.

3.6.2 ATP bioluminescence

Candida albicans cell suspensions of different concentrations were prepared as described in Section 3.6.1.1. One hundred microliter of the cell suspensions of different concentrations was added into each of 3 wells of an Opaque 96-well flat bottom assay plate. (Becton Dickinson, USA). One hundred microliter of

RPMI 1640 was added into 3 wells as the background control of the ATP bioluminescence measurement. One hundred microliter of the buffer solution of BacTiter-Glo™ Microbial Cell Viability Assay (Promega, USA) containing luciferin as substrate and luciferase was added to each well. The plate was covered with aluminum foil at room temperature for preventing the plate from illumination. The ATP bioluminescence was measured by Micro Lumat Plus Luminometer (EG and G Berthold, Germany) 10 minutes later. A graph of bioluminescence against the number of cells was plotted for the determination of the linear range and the detection limit of this method.

3.7 Effect of inoculum size on adhesion to catheters

3.7.1 Preparation of adhesion chambers

The adhesion chambers were made of 10g of high molecular weight agarose (USB, USA) dissolved in 200mL of double distilled water. The agarose was boiled using a household microwave oven until completely dissolved. Gel comb was loaded on a gel casting tray. The length of each well (adhesion chamber) was 0.5cm, the width was 0.3cm, and the depth was 1cm. The adhesion chambers were made on the day before the experiment to save time on the day of experiment. The whole gel tray was wrapped with parafilm, stored in a locally purchased plastic box at room temperature. The length of the box was 28cm, the width was 22cm, and the height

was 8cm. About 150mL of tap water was poured into the box for maintaining the humidity in the box. Two petri dishes were placed beneath the gel tray, preventing the gel tray from contacting the water.

3.7.2 Preparation of catheters

Tetrafluoroethylene and hexafloropropylene polymer catheters, polyurethane catheters, and silicone catheters were cut into 0.5cm fragments using a pair of sterile scissors, ruler and a pair of sterile forceps in a class II biosafety cabinet on the day before experiment to save time on the day of experiment. Twelve catheter fragments from each type of catheters were stored in 3 sterile plastic petri dishes at room temperature until use. These petri dishes were sealed with parafilm, wrapped by aluminum foil for preventing the catheter fragments from illumination. Catheter fragments were loaded into the adhesion chambers horizontally using a pair of sterile forceps in a class II biosafety cabinet just before the addition of *Candida albicans* cell suspensions.

3.7.3 Preparation of *Candida albicans* culture

Candida albicans culture was prepared as described in Section 3.4.1.2 except *Saccharomyces cerevisiae* was not cultured and the *Candida albicans* cell suspension was diluted to $2x10^9$cell/mL. One hundred microliter of the cell suspension was serially diluted with 900μl of YPD broth by every ten fold to

$2x10^5$cell/mL.

The cell concentration of each cell suspension was confirmed by viable count. Fifty microliter of each cell suspension was diluted with 950µl of PBS. One hundred microliter of the diluted cell suspensions of original cell concentration of $2x10^9$cell/mL, $2x10^8$cell/mL, $2x10^7$cell/mL, $2x10^6$cell/mL, and $2x10^5$cell/mL was further diluted serially in 900µl of PBS for 5, 4, 3, 2 and 1 times. One hundred microliter of each PBS diluted cell suspension was spread on a SDA. Three SDA were used for each cell suspension. The SDA were incubated at 30℃ for 48 hours. The number of CFU on SDA was counted. The cell concentration of each cell suspensions was calculated according to the following formula:

Cell concentration (cell/mL) = Number of CFU on SDA x Dilution Fold x 10

The factor of 10 was derived by the fact that only the cells from 100µl of each 1mL cell suspension was spread on SDA and subsequently counted.

3.7.4 Adhesion assay

After loading the catheter fragments in the adhesion chambers, 50µl of each *Candida albicans* cell suspension were added into the well of each type of catheters, three wells of each type of catheter for one suspension. Same volume of

YPD broth was added in 3 wells of each type of catheters as control of each type of catheter. The whole gel casting tray was wrapped by parafilm for preventing the evaporation of cell suspension during incubation. The adhesion chambers were incubated at 37℃ in 5% CO_2 for 30 minutes. Afterwards, catheter fragments were transferred aseptically in 36 new sterile 5mL screw capped glass bijou bottles, one catheter per bottle. The catheter fragments were washed with 200μl of PBS twice. The catheter fragments were placed into the wells of an opaque 96-well flat bottom plate. One hundred microliter of YPD broth was added to each well.

One hundred microliter of each *Candida albicans* cell suspension were added into the wells of the opaque 96-well flat bottom plate (Becton Dickinson, USA), 3 wells for one suspension, for the establishment of standard curve. One hundred microliter of YPD broth was added to 3 empty wells as the background control of the ATP bioluminescence measurement. One hundred microliter of the buffer solution containing luciferin as substrate and luciferase was added to each well with catheter fragments, to each well with the cell suspensions and to the background control wells. ATP bioluminescence measurement procedures were the same as described in Section 3.6.2 except that the cell concentration of standard curve was ranged from $2x10^9$cell/mL to $2x10^5$cell/mL instead of from $1x10^8$cell/mL to 1x10cell/mL. A graph of ATP bioluminescence against the number of cells was plotted for the establishment

of the standard curve. A line attaining the smallest sum of square of deviation was the line of best fit of the standard curve. The linear equation of the best fit line was determined by the following equation:

$$y = mx + C$$

where y is the ATP bioluminescence reading, x is the number of *Candida albicans* cells, m is the slope of the line of best fit, and C is the y-intercept of the line of best fit.

The number of *Candida albicans* cells adhered on each catheter fragments was calculated according to the linear equation of the corresponding standard curve of each type of catheters. The percentage of adhesion was calculated by the following equation:

$$\text{Percentage of adhesion} = \frac{\text{Number of cells on catheter}}{\text{CFU of corresponding initial inoculum}} \times 100\%$$

Two independent experiments were conducted on two different days for confirming the results.

3.8 Transformation of *Saccharomyces cerevisiae* with *ALS1*, *ALS5* smaller

allele, and *ALS6*

3.8.1 DNA extraction of *Candida albicans*

Candida albicans cells for DNA extraction was prepared as described in Section 3.5.1. The DNA of the cells was extracted by non-enzymatic lysis of *Candida albicans* cells and followed by protein precipitation by ammonium acetate and ethanol precipitation of DNA using MasterPure™ Yeast DNA Purification Kit (Epicentre, USA) according to manufacturer's instructions. Briefly, the pellet was resuspended in 300μl of Yeast Cell Lysis Solution. The microcentrifuge tube was incubated at 65℃ using a water bath for 15 minutes for the lysis of *Candida albicans* cells. The tube was placed on ice for 15 minutes. Then 150μl of MPC Protein Precipitation Reagent was added. The tube was vortex at the top speed of a Maxi Mix II Vortex Mixer (Thermolyne, USA) for 10 seconds. The tube was centrifuged at 22,713rcf for 10 minutes using the same centrifuge as above for precipitating proteins. The supernatant was transferred to a new 1.5mL microcentrifuge tube. Then 500μl of isopropanol (Beijing Reagent, China) was added for precipitating DNA. The tube was centrifuged at 22,713rcf for 10 minutes using the same centrifuge for harvesting DNA pellet. The supernatant was removed. The pellet was washed with 500μl of 70% ethanol (Merck, Germany). The tube was centrifuged at 22,713rcf for 10 minutes using the same centrifuge. The supernatant was removed. The DNA was dissolved in 50μl of

Ultrapure Distilled Water (Gibco, USA) at 72℃. The DNA was stored at -20℃.

Two microliter of the DNA extracted was pipetted into a quartz capillary tube (Amersham, USA) for measuring DNA concentration using GeneQuant RNA/DNA Calculator (Pharmacia, USA) at 260nm. DNA concentration of samples was calculated according to the formula:

DNA concentration (ug/mL) = Absorbance at 260nm x 50

3.8.2 PCR of *ALS1*, *ALS5* smaller allele, and *ALS6*

Each PCR consisted of 2μl of buffer of Platinum *Taq* High Fidelity polymerase (Invitrogen, USA), 0.8μl of 50mM magnesium chloride, 2μl of 2mM dNTP mix (Amersham, USA), 1μl of 10μM primer mix, and 2μl of 2ng/μl genomic DNA of *Candida albicans* strain SC5314 (ATCC® number: MYA-2876). Each PCR was made up to 20μl by Ultrapure Distilled Water (Gibco, USA). The reactions were heated to 94℃ for 2 minutes for activation of *Taq* polymerase. Thirty five cycles of PCR were performed using the following cycling parameters: 94℃ of denaturation for 10 seconds, 56℃ of annealing for 30 seconds and 68℃ of extension for 5 minutes. The PCR was completed after a final extension at 68℃ for 15 minutes. Five PCR were conducted for each gene and were pooled together after PCR for subsequent

manipulations.

Gel electrophoresis was performed as described in Section 3.5.2.2 except that 100μl of the PCR products of each *ALS* gene was mixed with 20μl of loading dye instead of mixing 20μl of RT-PCR products of each *ALS* gene with 4μl of loading dye.

3.8.3 Gel extraction

Agarose gel embedding the PCR products of each *ALS* gene was cut. The PCR products were extracted using QIAquick Gel Extraction Kit (Qiagen, USA) according to the manufacturer's instructions. Briefly, the agarose gel embedded with DNA was excised and weighted in four 1.5mL microcentrifuge tubes. One hundred microliter of sodium iodide containing Buffer QG was added to every 100mg of gel in each tube for dissolving the gel. The tubes were incubated at 50℃ using a water bath for 10 minutes. The solution was transferred to four of the spin columns provided. The columns were centrifuged at 22,713rcf for 1 minute. Then 500μl of Buffer QG was added to each column for removing remaining agarose gel. The columns were centrifuged at 22,713rcf for 1 minute using the same centrifuge. Then 750μl of Buffer PE was added to each column for washing the DNA recovered. The columns were centrifuged at 22,713rcf for 1 minute twice using the same centrifuge. PCR products were eluted in 40μl of Ultrapure Distilled Water (Gibco, USA) at 72℃. The concentration of the eluted PCR products was measured as described in Section 3.8.1.

3.8.4 Restriction digestion of PCR products of *ALS* genes and cloning plasmids

The restriction sites at the two termini of the PCR products of *ALS1* and the pYES6CT cloning plasmids (Invitrogen, USA) for ligating with *ALS1* were cut using BamH I and Not I (New England Biolabs, USA). *ALS5* smaller allele, *ALS6*, and the cloning plasmids for ligating with *ALS5* smaller allele, and *ALS6* were cut using Xba I and Not I (New England Biolabs, USA). Restriction digestion reactions of *ALS1*, and of pYES6CT cloning plasmid for ligating with *ALS1* consisted of 2μl of 10X NEBuffer 3, 0.2μl of 100X bovine serum albumin, 2μl of BamH I, 1μl of Not I, 10μl of purified *ALS1* PCR product or pYES6CT plasmid. Reactions were made up to 20μl by Ultrapure Distilled Water (Gibco, USA). The restriction digestion reactions of *ALS5* smaller allele, *ALS6*, and pYES6CT cloning plasmids for ligating with *ALS5* smaller allele, or *ALS6* consisted of 2μl of 10X NEBuffer 3, 0.2μl of 100X bovine serum albumin, 1μl of Not I, 1.5μl of Xba I, 10μl of purified *ALS5* smaller allele PCR product, purified *ALS6* PCR product, or pYES6CT plasmid. Reactions were made up to 20μl with Ultrapure Distilled Water (Gibco, USA).

The restriction digestions were conducted at 37℃ for 24 hours. Gel electrophoresis of restriction digestion products were performed as described in Section 3.5.2.2 for purification except that a 1% agarose gel was used instead of a

1.5% agarose gel. Restriction digestion products were extracted from the gel as described in Section 3.8.3. Concentration of the restriction digestion products extracted was measured as described in Section 3.8.1.

3.8.5 Ligation of *ALS1*, *ALS5* smaller allele, *ALS6* with pYES6CT cloning plasmids

The number of mole of the restriction digested PCR products of each *ALS* gene and of the cloning plasmid was calculated according to the formula:

$$\text{Number of mole (nmole)} = \frac{\text{Concentration of DNA (ng/µl)} \times \text{Volume of DNA (µl)}}{\text{Number of base of gene} \times 330}$$

The average molecular mass of nucleotide is 330

Molar ratio of the PCR products of each *ALS* gene to the cloning plasmids in the ligation was 4:1. Ligation reactions were catalysed by T4 DNA ligase (New England Biolabs, USA). Ligation of *ALS1* and pYES6CT cloning plasmid consisted of 2µl of T4 DNA ligase buffer, 1µl of T4 DNA ligase, 2µl of digested *ALS1*, 1µl of BamH I–Not I digested pYES6CT plasmid. The reaction was made up to 20µl with Ultrapure Distilled Water (Gibco, USA). Ligation of *ALS5* smaller allele, or *ALS6* with pYES6CT cloning plasmid consisted of 2µl of T4 DNA ligase buffer, 1µl of T4 DNA ligase, 2.36µl of digested *ALS5* smaller allele or *ALS6*, 1.2µl of Not I–Xba I digested

pYES6CT plasmid. The reaction was made up to 20μl with Ultrapure Distilled Water (Gibco, USA). Ligation was performed at 16℃ for 24 hours.

3.8.6 Transformation of ligated plasmids into *Escherichia coli*

Ligated plasmids were transformed into *Escherichia coli* of XL-10 Gold Ultracompetent Cells (Stratagene, USA) according to the instructions of the manufacturer. The *Escherichia coli* cells were derived from XL2-Blue strain. The genotype was Tet^r *D(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB* $lacI^qZDM15$ *Tn10* (Tet^r) Amy Cam^r]^a. Briefly, 4μl of β-mercaptoethanol provided in the kit was added to the *Escherichia coli* in each of three 1.5mL microcentrifuge tubes. The cells were placed on ice and were stirred every two minutes for five times. Two microliter of ligation products was added to the *Escherichia coli*. The cells were placed on ice for 30 minutes. The cells were heat shocked at 42℃ for exactly 30 seconds and were placed on ice for 2 minutes immediately after heat shock. Nine hundred microliter of NZY^+ broth was added to each tube of *Escherichia coli*. NZY^+ broth was prepared by dissolving 10g of casein hydrolysate (Fluka, Switzerland), 5g of yeast extract (Sigma, USA), 5g of sodium chloride (Merck, Germany) in 1L of double distilled water. The pH of the solution was adjusted to 7.5. The solution was autoclaved at 121℃, 103kPa for 15 minutes. The solution was mixed with 12.5mL of filter-sterile 1M magnesium chloride (Sigma,

USA), 12.5mL of filter-sterile 1M magnesium sulfate (Sigma, USA), and 20mL of filter-sterile 20% α-D-glucose (BDH, UK) aseptically. The tubes were incubated at 37℃ for 1 hour and shaked at 200r.p.m. One hundred microliter of the cells of each tube was plated on 3 LB agar with 50ug/mL ampicillin. Luria broth agar was prepared by dissolving 0.62g of luria broth base (Difco, USA), 0.8g of agar technical (Oxoid, UK) in 40mL of double distilled water. The agar was autoclaved at 121℃, 103kPa for 15 minutes. Two milligram of ampicillin (Sigma, USA) was added when the autoclaved agar was cooled to about 50℃. The cells were cultured at 37℃ for 24 hours.

3.8.7 Miniprep of plasmids

Five isolated colonies of transformed *Escherichia coli* of each *ALS* gene was subcultured in 8mL of LB broth with 50ug/mL of ampicillin in fifteen 15mL centrifuge tubes at 37℃ for 24 hours, 1 tube for a clone. The broth was centrifuged at 2016rcf for 5 minutes. The pellets were washed with 5mL of PBS twice. The plasmids of the *Escherichia coli* were extracted using QIAprep Spin Miniprep Kit (Qiagen, USA) by alkaline lysis of *Escherichia coli*, followed by the binding of DNA on silica membrane in acidic pH solution according to the instructions of manufacturer. The DNA was eluted in low salt solution or water (104, 105). Briefly, for each clone, the pellet was resuspended in 250μl of Buffer P1 of the kit and was transferred to a 1.5mL

microcentrifuge tube. Then 250µl of Buffer P2 was added and mixed by inverting the tube for 4 to 6 times. Then 350µl of Buffer N3 was added and mixed by inverting the tube for 4 to 6 times. The microcentrifuge tube was centrifuged at 22,713rcf for 10 minutes. The supernatant was transferred to a spin column provided. The column was centrifuged at 22,713rcf for 30 seconds. Then 500µl of Buffer PB was added to the column. The column was centrifuged at 22,713rcf for 30 seconds. Then 750µl of Buffer PE was added to the column. The column was centrifuged at 22,713rcf for 30 seconds twice. The plasmids were eluted with 50µl of Ultrapure Distilled Water (Gibco, USA) at 72℃. The concentration of the plasmids extracted was measured as described in Section 3.8.1. The plasmids were stored at -20℃.

3.8.8 DNA sequencing

DNA sequencing primers of the three genes were designed using software GeneTool™ (BioTools, Canada). The primers were designed according to the following criteria. First the T_M of the primers was between 50℃ to 60℃. Second, the distance between two successive sequencing primers of a gene was between 500 bases to 600 bases. Third, no false priming to other region of the gene was exhibited.

The sequences of the primers were shown in Appendix I. All the primers were purchased from Invitrogen, USA. Each of the primer powders was dissolved in Ultrapure Distilled Water (Gibco, USA) to the concentration of 100µM. Ten microliter

of the primer was diluted in 90µl of Ultrapure Distilled Water (Gibco, USA) as working stock (10µM).

DNA sequencing of the extracted plasmids was performed by Macrogen (South Korea) for confirming the sequences. Both of sense DNA strand and anti sense DNA strand of *ALS1, ALS5* smaller allele, *and ALS6* inserted into the plasmids were sequenced. The DNA sequences of the three *ALS* genes inserted into plasmids obtained by sequencing were aligned with the DNA sequences of the corresponding *ALS* gene sequence from GenBank of NCBI. Accession number of *ALS1* sequence was XM_712984 XM_436303, *ALS5* smaller allele sequence was AY227439, *ALS6* seuqence was AY225310.

Sequence variation found only on either sense DNA strand or anti sense DNA strand was considered to be a sequencing error. Sequence variation found on both strands was considered to be a true mutation.

3.8.9 Transformation of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae cells (ATCC® number: 96686) were made competent by lithium cations (106) using S.c. Easy comp Transformation Kit (Invitrogen, USA) according to the instructions of manufacturer. Briefly, 10mL of *Saccharomyces cerevisiae* cells was cultured in YPD broth until mid log phase. The cells were harvested and were washed with 10mL of Solution I of the kit. The cells

were centrifuged at 33.6rcf for 5 minutes at room temperature. The supernatant was removed. The pellet was resuspended in 1mL of Solution II of the kit. The cells were then competent and were used for transformation immediately.

Saccharomyces cerevisiae cells were transformed with the extracted plasmids by polyethylene glycol (107) using S.c. Easy comp Transformation Kit (Invitrogen, USA) according to the instructions of manufacturer. Briefly, 5µl of the plasmids was added to 50µl of competent *Saccharomyces cerevisiae* for transformation. Five hundred microliter of Solution III of the kit was mixed with the cells and the plasmids by vortex with the top speed of a Maxi Mix II Vortex Mixer (Thermolyne, USA). The cells were incubated at 30℃ using a water bath for 3 hours for transformation. The cells were vortexed every 15 minutes. Afterwards, the cells were centrifuged. Four hundred and fifty five microliter of the supernatant was removed. The pellets was resuspended in the residual fluid and spread on a yeast peptone dextrose agar with 25 µg/mL of blasticidin (YPDB). The agar was prepared by adding 1mL of 25mg/mL of blasticidin solution in 1L of autoclaved YPD agar at about 50℃. Yeast peptone dextrose agar was prepared as described in Section 3.1. Blasticidin solution was prepared by dissolving 25mg of blasticidin S HCl (Invitrogen, USA) in 1mL of Ultrapure Distilled Water (Gibco, USA).

The agar was incubated at 30℃ for 72 hours. The plates were sealed with

parafilm and were kept at 4℃ for long term storage. A single isolated colony of transformed *Saccharomyces cerevisiae* of each of the three *ALS* genes was picked and subcultured on another 4 YPDB. *ALS1*, *ALS5* smaller allele, *ALS6*, or empty plasmid transformed *Saccharomyces cerevisiae* used in future experiments were picked from these plates (master plates).

3.8.10 Detection of Als1, Als5, and Als6 proteins expression

3.8.10.1 Preparation of cultures in SC synthetic medium

Each *Saccharomyces cerevisiae* clone was picked from corresponding master plates and was subcultured on another 4 YPDB. The agar was cultured at 30□ for 48 hours. A single isolated colony of each of the 4 clones was subcultured in 50mL of SC synthetic medium with 2% glucose and 25μg/mL blasticidin at 30℃ for 48 hours without shaking. The absorbance of the *Saccharomyces cerevisiae* cultures at 600nm was measured. The OD of each of 100mL of fresh SC synthetic medium with 3% galactose and 25mg/mL blasticidin was adjusted to 0.4 by adding an appropriate volume of the overnight cultures. The volume of cell suspensions needed was calculated as described in Section3.4.1.2. The overnight culture of each clone was centrifuged at 3528rcf for 5 minutes. The supernatant was removed. The pellets were washed with 5mL of PBS twice before resuspended in 100mL of SC synthetic medium with 3% galactose and 25μg/mL blasticidin. The SC synthetic medium with

3% galactose and 25mg/mL blasticidin was incubated at 30℃ for 18 hours without shaking.

3.8.10.2 Protein extraction

The cells were centrifuged at 6272rcf for 5 minutes. The supernatant was removed. The pellets were resuspended in 1mL of 1.14% ammonium carbonate. Fifty microliter of 100mM phenylmethysulfunyl fluoride (PMSF) (Roche, USA) and 2µl of β-mercaptoethanol (Merck, Germany) were added. The cells were transferred to 4 glass test tubes. One millilitre of glass beads (Sigma, USA) was added to each of the test tubes. The top of the test tubes were wrapped with parafilm for prevention of spilling during vortex. The test tubes were vortexed at the top speed of a Maxi Mix II Vortex Mixer (Thermolyne, USA) for 1 minute and then placed in ice for 1 minute before another vortexing. The test tubes were vortexed for five times. The cell lysates were transferred to four 1.5mL microcentrifuge tubes and centrifuged at 1209rcf for 10 minutes. The supernatant was removed. The pellets were washed with 1mL of 1.14% ammonium carbonate twice. The pellets were resuspended in 1mL of 1.14% ammonium carbonate. Fifty microliter of 100mM PMSF and 2µl of β-mercaptoethanol were added to prevent the proteins from degradation. The cell wall extracts were stored at -80℃.

3.8.10.3 Dot blot of cell wall lysates

A piece of PVDF membrane (BioRad, USA) with the length of 10cm, and width of 7cm was soaked in 20mL of methanol until the membrane was transparent in a locally purchased plastic box. The length of the plastic box was 12cm, the width was 8cm, and the height was 3cm. The methanol was removed. The membrane was washed with 10mL of double distilled water. Ten milliliter of double distilled water was poured in another plastic box of the same size. The membrane was transferred to this plastic box. The double distilled water was added to prevent the membrane from drying before the dotting of cell wall extracts. Ten microliter of the cell wall extracts from the *Saccharomyces cerevisiae* cells transformed with *ALS1*, *ALS5* smaller allele, and *ALS6* was dotted on the PVDF membrane and air-dried. The membrane was soaked in 10mL of PBSTM and was shaked at 100r.p.m. for one hour. The PBSTM was removed. The membrane was washed with 10mL of PBST and was shaked at 100r.p.m. for 5 minutes. The washing step was repeated. The PBST was removed. The membrane was soaked in 10mL of PBSTM. Two microliter of anti-His antibody was added in the PBSTM. The membrane was shaked at 100r.p.m. for 24 hours. The PBSTM with antibodies in it was removed. The membrane was washed with 10mL of PBST and was shaked at 100r.p.m. for five minutes. The washing step was repeated. The membrane was transferred to a swan warp with the side added with cell wall extracts on top. One millilitre of Reagent 1 and 1mL of Reagent 2 of ECL Western

Blotting Reagent (Amersham, USA) were mixed in a sterile 5mL screw capped glass bijou bottle. The mixed blotting reagent was applied over the surface of the membrane. The membrane was wrapped by the swan warp. The membrane and a medical imaging film (Fuji, Japan) were placed in a film holder together in a dark room for exposure for 20 minutes. The film was developed.

3.9 Adhesion of transformed *Saccharomyces cerevisiae* to fibroblasts

3.9.1 Preparation of fibroblasts and of *Saccharomyces cerevisiae* cultures

The procedures of the preparation of fibroblasts substratum prepared as described in Section 3.4.1.1.

The cultures of transformed *Saccharomyces cerevisiae* of *ALS1*, *ALS5* smaller allele, *ALS6*, and empty plasmid (as control) were prepared as described in Section 3.8.10.1 except that 15mL, instead of 50mL of SC synthetic medium with 2% glucose and 25μg/mL of blasticidin was used for the subculture of isolated colony of each clone, and 30mL, instead of 100mL of SC synthetic medium with 3% galactose and 25μg/mL of blasticidin was used for the subculture of *Saccharomyces cerevisiae* cells harvested from SC synthetic medium with 2% glucose and 25μg/mL of blasticidin. After 18 hours of incubation, the medium was centrifuged at 3528rcf for 5 minutes. The pellets were washed with 5mL of PBS twice. The pellets were resuspended in 5mL of SC synthetic medium with 3% galactose. The number of

Saccharomyces cerevisiae cells in the medium was determined using a Fuchs-Rosenthal counting chamber. The cell suspensions were diluted to 300cell/mL with SC synthetic medium supplemented with 3% galactose. The inoculum size was confirmed by viable count. Two hundred microliter of the cell suspensions was spread on a SDA. Three SDA were used for one cell suspension. The SDA were incubated at 30℃ for 48 hours. The number of CFU on the SDA was counted. The number of *Saccharomyces cerevisiae* cells in 1mL of the cell suspensions was calculated according to the following formula:

Number of cells per mL = (Average of number of CFU on 3 SDA) x 5

The factor of 5 was derived by the fact that only the cells in 200µl of the 1mL cell suspensions were counted.

3.9.2 Adhesion assay

The adhesion assay was conducted as described in Section 3.4.1.3 except cell suspensions of transformed *Saccharomyces cerevisiae* were used instead of *Candida albicans* cell suspension.

3.10 Adhesion of transformed *Saccharomyces cerevisiae* to FEP, polyurethane, and silicone catheters

3.10.1 Preparation of catheters, adhesion chambers, and transformed *Saccharomyces cerevisiae* cultures

Catheter fragments and adhesion chambers were prepared as described in Section 3.7.1 and Section 3.7.2.

Saccharomyces cerevisiae cultures of each clone were prepared as described in Section 3.9.1 except that the cell suspensions of each clone were diluted to $2x10^8$cell/mL with SC synthetic medium with 3% galactose.

One hundred microliter of the *Saccharomyces cerevisiae* cell suspensions of each clone was serially diluted with 900µl of SC synthetic medium with 3% galactose by every ten fold to 2x10cell/mL for the establishment of standard curve. The cell concentration of each diluted cell suspension was confirmed by viable count. The viable count was conducted as described in Section 3.7.3 except that viable count of cell suspension at concentration of $2x10^9$cell/mL was not performed.

3.10.2 Adhesion to catheter fragments

The adhesion assay was conducted as described in Section 3.7.4 except fifty microliter of the $2x10^8$cell/mL culture of each transformed *Saccharomyces cerevisiae* (total $1x10^7$ cells) were added instead of *Candida albicans* cell suspensions. And same volume of SC synthetic medium with 3% galactose was added in 3 well of each type of catheters as control, instead of using YPD broth as control. ATP

measurement procedures were the same as described in Section 3.7.4 except that measurement of cell suspension at the concentration of $2x10^9$cell/mL was not performed. The standard curve was ranged from $2x10^8$cell/mL to 2x10cell/mL.

3.11 Statistical analysis

All the statistical analysis was performed using Statistical Package for Social Sciences 14.0 for Windows (SPSS inc, USA). Mann-Whitney U test was used to compare the percentage of adherence of *Candida albicans* and *Saccharomyces cerevisiae* towards fibroblasts. Nonparametric ANOVA, Kruskal-Wallis test was used for comparing the result of percentage of adherence of *Candida albicans* towards catheters at different cell concentrations, and to compare the percentage of adherence of *Saccharomyces cerevisiae* transformed with different *ALS* genes towards fibroblasts and to catheters.

Chapter 4: Results

4.1 Confirmation of identity of *Candida albicans* and of *Saccharomyces cerevisiae*

The identity of presumptive *Candida albicans* was confirmed by morphology test on cornmeal agar, germ tube test, biochemical tests (carbohydrate assimilation tests), and coloration of colony on chromogenic agar. The identity of presumptive *Saccharomyces cerevisiae* was confirmed using the methods stated above except the coloration of colony.

4.1.1 *Candida albicans*

In the morphology test on cornmeal agar, hyphae was observed. Clusters of blastoconidia at the septa of hyphae, and single chlamydospore at the terminus of hyphae were observed. In the germ tube test, germ tubes extending from oval yeast cells without constriction at point of origin were observed after incubation of *Candida albicans* in serum for 2 hours at 37℃. In biochemical tests, the profile index obtained indicates 99.4% probability of the yeast being a *Candida albicans*. In the coloration of colony test, the coloration of colony of the yeast was green.

4.1.2 *Saccharomyces cerevisiae*

In the morphology test on cornmeal agar, large blastoconidia with few pseudohyphae were observed on cornmeal agar. No hyphae or chlamydospore was

observed on cornmeal agar. In the germ tube test, no germ tube was observed after incubation in serum for 3 hours at 37℃. In biochemical tests (carbohydrate assimilation tests), the profile index indicated 99.3% probability of the yeast being a *Saccharomyces cerevisiae*.

4.2 Cell culture of fibroblasts

The morphology and confluence level of fibroblasts cultured in T_{25} culture flask after recovery was checked after incubation at 37℃ in 5% CO_2 and 95% RH for 72 hours. A monolayer of fibroblasts of 90% confluence was observed. Spindle-like structures extending from cell were observed from each cell (Figure 1a). This morphology was similar to the morphology of the fibroblasts described by ATCC (Figure3.1b). As the recovered fibroblasts attained 90% confluence after incubation at 37℃ in 5% CO_2 and 95% RH for 72 hours, the fibroblasts were subcultured into a T_{75} culture flask for establishment of cell line.

4.3 Preliminary studies of adherence of *Candida albicans* to fibroblasts and to FEP, polyurethane, and silicone catheters

4.3.1 Adherence to fibroblasts

The confluence of fibroblasts cultured in a T_{75} culture flask was checked after incubation at 37℃ in 5% CO_2 and 95% RH for 48 hours. A monolayer of fibroblasts of 80% confluence was observed. The fibroblasts were harvested and

counted. After counting, $2x10^6$ cells were seeded in each well of two 6-well plates. The confluence of fibroblasts cultured in each well of the two culture plates was checked. A monolayer of fibroblasts of 100% confluence was observed (Figure 2). The cells were used as substratum for preliminary adhesion assay of *Candida albicans* to fibroblasts.

The adherence of *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) was compared with that of *Saccharomyces cerevisiae*, which was known to be non adherent. The adherence of *Candida albicans* and that of *Saccharomyces cerevisiae* was represented by percentage of initial inoculum adhered on fibroblasts. The percentage of initial inoculum of *Saccharomyces cerevisiae* adhered to fibroblasts was 0.29%. The percentage of initial inoculum of *Candida albicans* adhered to fibroblasts was 6.40% (Figure 3). The statistical significance of the difference in the percentage of initial inoculum was tested by Mann-Whitney U test. The difference was statistically significant with p value = 0.04.

4.3.2 Adherence to catheters

Adherence of *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) to FEP, polyurethane, and silicone catheter fragments were studied by the turbidity of YPD broth culturing catheter fragments after adhesion of *Candida albicans* and by the observation of catheter fragments, except silicone catheter

fragments , stained with acridine orange, using a phase contrast microscope darkfield microscope after adhesion of *Candida albicans*.

The turbidity of YPD broth culturing the FEP, polyurethane, and silicone catheter fragments after adhesion of *Candida albicans* was compared visually with that of YPD broth culturing the catheter fragments of the control group. The YPD broth culturing the FEP, polyurethane, and silicone catheter fragments after adhesion of *Candida albicans* was more turbid than those of the control group (Table 1).

Green fluorescence was observed on the surface of FEP catheter fragments of the control group. Green fluorescence was not observed on the surface of FEP catheter fragments after adhesion of *Candida albicans*. Orange spots were observed on the surface of FEP catheter fragments after adhesion of *Candida albicans*.

Green fluorescence was observed on the surface of polyurethane catheter fragments of the control group. Green fluorescence was observed on the surface of polyurethane catheter fragments after adhesion of *Candida albicans*. Yellow spots were observed on the surface of polyurethane catheter fragments (Figrue 4).

4.4 Confirmation of expression of *ALS1, ALS5* smaller allele, and *ALS6* of *Candida albicans* in YPD broth

Expression of *ALS1*, *ALS5* smaller allele, and *ALS6* in *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) cultured in YPD broth was confirmed by

RT–PCR using RNA extracted from cells of *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) cultured in YPD broth. The coding DNA (cDNA) products were loaded into an agarose gel for gel electrophoresis. No cDNA product was present in the negative control of RT-PCR reactions of the three *ALS* genes. No PCR product was present in the PCR reaction of the three *ALS* genes using the extracted RNA as template. Coding DNA products were observed in the RT-PCR reactions of the three *ALS* genes (Figure 5).

4.5 Establishment of quantitation system of adhesion assay

4.5.1 Absorbance measurement of *Candida albicans* stained with safranin

From the plot of absorbance of SDS buffer against cell number (Figure 6), the background absorbance of the SDS buffer was 0.269. The absorbance reading in the range of 1 cell to $1x10^4$ cells fluctuated between 0.241 and 0.301. The absorbance readings were not differentiable in this range. The absorbance reading increased from $1x10^4$ cells. The absorbance reading at $1x10^4$, $1x10^5$ and $4x10^5$ cells was 0.27, 0.37, and 0.42 respectively. The absorbance reading increased at a higher rate from $4x10^5$ cells to $1x10^7$ cells. The absorbance reading at $1x10^7$ cells was 0.96. The linear range was between $4x10^5$ cells to $1x10^7$ cells

4.5.2 ATP bioluminescence

From the plot of log (signal to noise ratio) against log (cell number)

(Figure 7), the log (signal to noise ratio) increased from 1x10 to $1x10^3$ cells at a slower rate. The log (signal to noise ratio) of 1x10, $1x10^2$, and $1x10^3$ cells was 0, 0.61, and 0.60 respectively. The log (signal to noise ratio) increased at a faster rate from $1x10^3$ to $1x10^7$ cells. The log (signal to noise ratio) of $1x10^4$, $1x10^5$, $1x10^6$, and $1x10^7$ cells was 1.32, 2.19, 3.25, and 4.10 respectively. The log (signal to noise ratio) dropped to 3.71 at $1x10^8$ cells. The log (signal to noise ratio) value of the detection limit of the ATP bioluminescence assay was set to be two standard $\text{deviation}_{(\text{background})}$ above the background. The log (signal to noise ratio) value of the detection limit was 1.3, corresponding to $1x10^4$ cells.

4.6 Effect of inoculum size on adhesion to catheters

The effect of inoculum size on adhesion to FEP, polyurethane, and silicone catheter fragments was tested using $1x10^5$, $1x10^6$, and $1x10^7$ cells of *Candida albicans* strain SC5314 (ATCC® number: MYA-2876). The adherence of *Candida albicans* to catheter fragments was represented by percentage of initial inoculum adhered to catheters. A graph of percentage of initial inoculum adhered to catheter against log (number of cells) of each catheter was plotted (Figure 8).

The percentage of initial inoculum of *Candida albicans* adhered to FEP catheter fragments decreased as the cell number increased. The percentage of initial inoculum of *Candida albicans* adhered to FEP catheter fragments at $1x10^5$, $1x10^6$, and

$1x10^7$ cells was 1.66%, 0.51%, and 0.24% respectively. The statistical significance of the difference in percentage of initial inoculum was tested by non parametric ANOVA, Kruskal-Wallis test. The p value was 0.113 and the difference was not statistically significant.

The percentage of initial inoculum of *Candida albicans* adhered to polyurethane catheter fragments at $1x10^5$, $1x10^6$, and $1x10^7$ cells was 0.76%, 0.23%, and 0.31% respectively. The statistical significance of the difference in percentage of initial inoculum was tested by non parametric ANOVA, Kruskal-Wallis test. The p value was 0.193 and the difference was not statistically significant.

The percentage of initial inoculum of *Candida albicans* adhered to silicone catheter fragments decreased as the cell number increased. The percentage of initial inoculum of *Candida albicans* adhered to silicone catheter fragments at $1x10^5$, $1x10^6$, and $1x10^7$ cells was 2.45%, 1.34%, and 0.51% respectively. The statistical significance of the difference in percentage of initial inoculum was tested by non parametric ANOVA, Kruskal-Wallis test. The p value was 0.177 and the difference was not statistically significant.

4.7 Transformation of *Saccharomyces cerevisiae* with *ALS1*, *ALS5* smaller allele, and *ALS6*

4.7.1 PCR of *ALS1*, *ALS5* smaller allele, and *ALS6*

The three *ALS* genes were first amplified from the genomic DNA of the *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) by PCR using primers with restriction digestion sites at the termini. The reaction mixtures of the three *ALS* genes after PCR were loaded in an agarose gel for gel electrophoresis. No PCR product was present in the negative control of the PCR reactions of the three *ALS* genes. Single band was observed for the PCR of each *ALS* genes. The size of the PCR products were between 3054bp and 4072bp (Figure 9).

4.7.2 Ligation of PCR products with pYES6CT plasmids

Restriction digestion products of the PCR products of *ALS1*, *ALS5* smaller allele, and *ALS6* were ligated with digested pYES6CT plasmids. Presence of *ALS* genes in the ligated plasmids was confirmed by PCR of the *ALS* genes from the three ligated plasmids. The same primers of the PCR reactions of the three *ALS* genes were used. The reaction mixtures were loaded in an agarose gel for gel electrophoresis. No PCR product was present in the negative control of the PCR reactions of the three *ALS* genes. No PCR product was present in the controls of the three *ALS* genes using original pYES6CT plasmid as template. Smear from the top of the gel with a single band was observed for the PCR reactions of *ALS1*, and *ALS5* smaller allele using *ALS1*, and *ALS5* ligated plasmids as template. Single band was observed for the PCR of *ALS6* using *ALS6* ligated plasmid as template. The band intensity of the PCR

product of *ALS6* amplified from ligated plasmid was weaker than that of *ALS1* and *ALS5* smaller allele. The band size of the PCR products amplified from the three ligated plasmids was the same as that from the genomic DNA of *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) (Figure 10).

4.7.3 DNA sequencing results of *ALS1*, *ALS5* smaller allele, and *ALS6* ligated plasmids

The *ALS* genes inserted into plasmids were sequenced by the DNA sequencing services of Macrogen, South Korea. The DNA sequences of the *ALS* genes inserted were compared with the DNA sequences of its corresponding gene retrieved from the GenBank of NCBI. The DNA sequences of the *ALS1*, *ALS5* smaller allele, and *ALS6* genes inserted were shown in appendix II, appendix III, and appendix IV resepctively. A base variation in *ALS1* was observed at the position of 1933rd bp. The original cytosine changed into thymine, leading to a change in amino acid from leucine to phenylalaine. Two base variations in *ALS5* smaller allele were observed at the positions of 48th bp, and of 258th bp. The thymine at the 48th bp changed into cytosine, and the guanine at the 258th bp changed into adenine. Both variations were synonymous in amino acid level. A base variation in *ALS6* was observed at the position of 3529th bp of the *ALS6*. The adenine at the 3529th bp changed into guanine, leading to a change of amino acid from threonine to alanine. The base variations of

each gene, with the positions of variations and the amino acid changes were listed in Table 2.

4.7.4 Detection of Als1, Als5, and Als6 proteins expression

Expression of Als1, Als5, and Als6 proteins in transformed *Saccharomyces cerevisiae* was checked by dot blot. Anti-His HRP antibodies bound to the His-tag at the tail of each Als protein. Chemiluminescence generated from anti-his HRP leaving dark spots on medical imaging film. From the film (Figure 11) dark spot was not present in the region corresponding to the PVDF membrane dotted with the cell wall lysate of *Saccharomyces cerevisiae* transformed with empty plasmid. Dark spots were detected in the region corresponding to the PVDF membrane dotted with the cell wall lysates of *Saccharomyces cerevisiae* transformed with *ALS1*, *ALS5* smaller allele and *ALS6*.

4.8 Adhesion of transformed *Saccharomyces cerevisiae* to fibroblasts

The confluence of fibroblasts cultured in a T_{75} culture flask was checked after the incubation at 37℃ in 5% CO_2 and 95% RH for 48 hours. A monolayer of fibroblasts of 80% confluence was observed. The fibroblasts were harvested and counted. After counting, $2x10^6$ cells were seeded in each well of two 6-well plates. The confluence of fibroblasts cultured in each well of the two culture plates was checked. A monolayer of fibroblasts of 100% confluence was observed. The cells

were used as substratum for the adhesion assay of *Saccharomyces cerevisiae* to fibroblasts.

The adherence of the *Saccharomyces cerevisiae* transformed with *ALS1*, *ALS5* smaller allele, or *ALS6* was compared with that of *Saccharomyces cerevisiae* transformed with empty pYES6CT plasmid (control). The adherence of the four clones of *Saccharomyces cerevisiae* was represented by percentage of initial inoculum adhered to fibroblasts.

The percentage of initial inoculum of the three clones of *ALS* transformed *Saccharomyces cerevisiae* was higher than that of control. The percentage of initial inoculum adhered to fibroblasts of control, and of *Saccharomyces cerevisiae* transformed with *ALS1*, *ALS5* smaller allele, and *ALS6* was 10.36%, 44.87%, 24.02%, and 27.69% respectively (Table 3 and Figure 12). The statistical significance of the difference in the percentage of initial inoculum of the four clones of *Saccharomyces cerevisiae* was tested by non parametric ANOVA, Kruskal-Wallis test. The p value was 0.04. The percentage of initial inoculum adhered to fibroblast of all the three clones of *ALS* transformed *Saccharomyces cerevisiae* was significantly higher than that of control.

4.9 Adhesion of transformed *Saccharomyces cerevisiae* to FEP, polyurethane and silicone catheters

The adherence of the *Saccharomyces cerevisiae* transformed with *ALS1*, *ALS5* smaller allele, and *ALS6* was compared with that of *Saccharomyces cerevisiae* transformed with empty pYES6CT plasmid (control). The adherence of the four clones of *Saccharomyces cerevisiae* was represented by percentage of initial inoculum adhered to the catheter fragments.

The percentage of initial inoculum adhered to FEP catheter fragments of the three clones of *ALS* transformed *Saccharomyces cerevisiae* was higher than that of control. The percentage of initial inoculum adhered to FEP catheter fragments of control, and of *Saccharomyces cerevisiae* transformed *ALS1*, *ALS5* smaller allele, and *ALS6* was 0.17%, 3.93%, 0.37%, and 1.01% respectively. The statistical significance of the difference in the percentage of initial inoculum of the four clones of *Saccharomyces cerevisiae* was tested by non parametric ANOVA, Kruskal-Wallis test. The p value was 0.041. The difference in percentage of initial inoculum between control and *ALS1*, and *ALS6* transformed *Saccharomyces cerevisiae* was statistically significant. The percentage of initial inoculum of the *Saccharomyces cerevisiae* transformed with *ALS5* smaller allele was not significantly higher than that of control.

The percentage of initial inoculum adhered to polyurethane catheter fragments of the *Saccharomyces cerevisiae* transformed with *ALS1*, and with *ALS5* smaller allele was higher than that of control. The percentage of initial inoculum

adhered to polyurethane catheter fragments of *ALS6* transformed *Saccharomyces cerevisiae* was below the detection limit of the quantitation system. The percentage of initial inoculum adhered to polyurethane catheter fragments of control, and *Saccharomyces cerevisiae* transformed with *ALS1*, and *ALS5* smaller allele was 0.16%, 0.28%, and 0.29% respectively. The statistical significance of the difference in the percentage of initial inoculum between the three clones of *Saccharomyces cerevisiae* was tested by non parametric ANOVA, Kruskal-Wallis test. The p value was 0.276 and the difference was not statistically significant.

The percentage of initial inoculum adhered to silicone catheter fragments of the *ALS1* transformed *Saccharomyces cerevisiae* was higher than that of control. The percentage of initial inoculum adhered to silicone catheter fragments of the *Saccharomyces cerevisiae* transformed with *ALS5* smaller allele was lower than that of control. The percentage of initial inoculum of *ALS6* transformed *Saccharomyces cerevisiae* was below the detection limit of the quantitation system. The percentage of initial inoculum adhered to silicone catheter fragments of control, and of *Saccharomyces cerevisiae* transformed with *ALS1*, and *ALS5* smaller allele, was 0.47%, 2.57%, and 0.19% respectively. The statistical significance of the difference in the percentage of initial inoculum between the three clones was tested by non parametric ANOVA, Kruskal-Wallis test. The p value was 0.768 and the difference

was not statistically significant. The percentage of initial inoculum of the four clones of *Saccharomyces cerevisiae* adhered to the FEP, polyurethane, and silicone catheters was shown in Table 4 and Figure 13.

Chapter 5 Discussion

5.1 Limitations of static adhesion assay model

Static adhesion model inherits the limitation of the domination of gravitational force as stated in Section 1.7.1. In addition, the shearing effect of fluid movement on adhesion of microbial cells cannot be studied by static adhesion model.

In this study, the use of catheter tubes instead of catheter material discs prohibits direct comparison of the adhesive function of Als proteins to different types of catheter. The geometry of the FEP, the polyurethane, and the silicone catheters used is different as their diameters are different. This affects the probability of *Saccharomyces cerevisiae* cells contacting the catheter surfaces, therefore direct comparison of adherence between the three catheters is inappropriate. This problem can be solved by using catheter material sheets, which can be tailored into disc of same diameter, in turn, the geometry and the surface area. The use of catheter material disc offers another advantage of the ease of conducting adhesion assay. Microtiter plates could be used as adhesion chambers if catheter material discs were used. All the cell suspensions applied would be on the top of the disc and cells could sediment on and contact the surface of the discs. Adhesion chambers had to be tailor-made, which was a tedious work, as catheters were used in this study. However, it is very difficult to obtain catheter material sheets due to the unwillingness of catheters manufacturers

providing the services, the cost involved, and, the difficulties in transportation of these sheets.

Adherence of different Als proteins is reflected by the percentage of initial inoculum remaining on catheter surfaces after the washing process. Ideally, the number of *Saccharomyces cerevisiae* cells adhered on catheter surfaces after washing should be the function of the adhesive strength between the cells and catheter surfaces. However, as in most adhesion studies, the actual shearing force exerted on cells during washing was not defined and controlled. In addition, there is no standard on the strength of shearing force for adhesion assay. Without the information of the absolute adhesive strength between the cells and catheter surfaces, the result obtained from this type of adhesion assay is relative in nature. Therefore, direct comparison between different studies is inappropriate unless the washing process is similar.

5.2 Quantitation System

5.2.1 Staining method

The detection limit of the staining method using safranin was $4x10^5$ cells. If 10^7 cells were used as the initial inoculum of an adhesion assay, the detection limit in terms of percentage of adherence would be 4%. From a number of adhesion assay, the percentage of initial inoculum adhered to substrata could be lower than 1% (86). Thus this method is not sensitive enough. This method originates from the

measurement of biofilm by staining the extracellular matrix of *Staphylococcus aureus* with safranin stain (92). One of the probable reasons explaining the high detection limit would be the inability of *Candida albicans* cell wall in absorbing safranin stain. Incomplete destaining by SDS buffer may also account for the high detection limit.

5.2.2 ATP bioluminescence assay

The detection limit of the ATP bioluminescence assay was $1x10^4$ cells or 0.1% in terms of percentage of adherence if $1x10^7$cells were used as initial inoculum. As the detection limit of this assay was 40 times lower than that of the staining method and lower than 1%, ATP bioluminescence assay was used in this study.

Detection limit of ATP bioluminescence is defined by the following equation (108):

Detection limit = Average Relative Luminescence Unit (RLU) $_{(background)}$ + 2 x Standard Deviation $_{(background\ RLU)}$

The strategy to lower the detection limit is to decrease the background RLU and to minimize the variation of background RLU, which will be small if the background RLU is low. The background RLU was contributed by the autoluminescence of the 96-well white assay plates used, the ATP from the medium submerging the catheter fragments during measurement, and background contamination.

Autoluminescence of the white assay plate is due to the emission of energy

by the plate after absorbing light. This was prevented by wrapping the white assay plates with aluminum foil before use. Yeast peptone dextrose broth and RPMI 1640 were used in the ATP bioluminescence assay. They carried ATP molecules of the raw materials of the medium. It may be prevented by using autoclaved distilled water instead of using medium for submerging catheters. A major source of background ATP contamination was from the white assay plates as the plates used were not sterile. Microbial cells in the wells of the plates increased background signal. Effort was made for obtaining sterile white assay plates but was unsuccessful. In addition, the adhesion assay of catheters involved a lot of transfer of catheter fragments between bijou bottles. These manipulations increased the chance of background contamination although sterile forceps were used. It can be improved if catheter material discs were used.

The bioluminescence reading decreased at cell concentration of 10^8 cells. One possible reason is that cell suspension at this concentration was too turbid and blocking the emission of bioluminescence. The upper limit of this system was 10^7 cells as bioluminescence was not proportional to cell number beyond 10^7 cells.

This quantitation system is useful in studying initial adhesion events because the initial inoculum size of this kind of study is usually above $1x10^6$ cells. Therefore, the minimum detection limit of this quantitation system in terms of

percentage of initial inoculum would be lower than 1%, which is sufficient to detect low level of adherence. In addition, this system is less tedious, and faster than other quantitation systems such as sonication and staining, especially if catheter material disc is used. The system may also be useful in the study of cell proliferation after adhesion. Although the initial inoculum of this kind of study is lower than $1x10^6$ cells, thus the detection limit of this assay, the number of cells after the long incubation time may be well above $1x10^7$ cells. However, biofilm formation is very likely to occur after the long incubation time, and the effect of biofilm on the accuracy of the quantitation system is unknown. As a result, further experiments have to be conducted in order to verify this possible use of the system. Clinical use of this quantitation system may not be possible. Quantitation of microorganisms adhered on catheters by ATP bioluminescence will kill the microorganisms and the identification of causative agents will not be possible. In addition, as a standard curve has to be constructed for each species or even each strain of microorganisms in bioluminescence assay, this may not be possible to conduct in a clinical microbiology laboratory.

5.3 Preliminary studies of adherence of *Candida albicans* to fibroblasts and to FEP, polyurethane, and silicone catheters

The aim of this study is to confirm the adherence of *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) to fibroblasts and to the three catheters

tested before cloning of the *ALS* genes of this strain. Adherence of *Candida albicans* strain SC5314 to fibroblasts was compared with that of *Saccharomyces cerevisiae* (ATCC® number: 96686), which is known to be non adherent. The adherence of the *Candida albicans* to fibroblasts was 22.3 folds higher than that of the *Saccharomyces cerevisiae*. Thus it was believed that *Candida albicans* strain SC5314 is more 'adherent' to the fibroblasts.

In the preliminary study of the adherence of *Candida albicans* strain SC5314 to the three catheters, *Candida albicans* cells were observed on FEP and on polyurethane catheter surfaces. In addition, growth of *Candida albicans* cells was observed in the YPD broth culturing FEP, polyurethane, and silicone catheters, but not in the catheters of the control group. It was believed that *Candida alibcans* cells adhered to the three catheters. As this *Candida albicans* strain is 'adherent' to fibroblasts and to catheters, the *ALS* genes from this strain were cloned.

5.4 Effect of inoculum size on adhesion to catheters

The aim of this experiment is to determine the inoculum size to be used for adhesion assay of catheters. The detection limit of the ATP bioluminescence assay in terms of percentage of initial inoculum adhered to catheters can be lowered by increasing the initial inoculum size. However, catheter surfaces may be saturated if the inoculum size was too high. This may underestimate the adherence of *Candida*

albicans or of transformed *Saccharomyces cerevisiae* as cells could not contact the catheter surfaces (36). Assuming the inherent adherence of *Candida albicans* cells is not affected by the cell density in the surrounding fluid, the percentage of adherence of *Candida albicans* using different initial inoculum size should be the same if the catheters were not saturated.

No statistically significant difference in percentage of initial inoculum adhered on catheters was detected using $1x10^5$, $1x10^6$, and $1x10^7$ cells as initial inoculum for the three catheters. It suggests that the catheter fragments were not saturated. However, an increasing trend in the percentage of adherence was observed for all the three catheters as the initial inoculum size decreased. A statistically significant result may be obtained if more replicates were conducted. It should also be noted that the adhesive strength of the *Candida albicans* cells may be weak and most of the cells were washed away. Statistically significant difference may not be detected if too few cells remaining on catheters.

As there was no statistically significant difference in the percentage of initial inoculum adhered on the three catheters at different inoculum size, the inoculum size was set at $1x10^7$ cells in order to minimize the detection limit of the ATP bioluminescence assay in terms of percentage of adherence of the ATP bioluminescence assay.

5.5 Selection of *ALS* genes

Saccharomyces cerevisiae was originally transformed with *ALS1*, *ALS3*, *ALS5*, and *ALS6*. *Saccharomyces cerevisiae* was not transformed with *ALS3* because *ALS3* gene could not be amplified from the genomic DNA of *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) after many effort. *ALS1*, *ALS3*, and *ALS5* were selected because they exhibited adhesion properties to a large array of substrata (86). It is likely that they also exhibit adhesion properties to fibroblasts although its adherence to FEP, polyurethane, and silicone catheters were not predictable. In addition, since the 5' domains of the three genes which were postulated responsible for the adhesion properties share 85% of sequence identity (81, 83), discrepancy in the adhesion properties between the three genes to either fibroblasts or catheter materials may further support the hypothesis that variations in the hypervariable region of the three genes account for the different substrate specificity (86). *ALS6* was selected for study because its adhesion property was different from that of *ALS1*, *ALS3*, and *ALS5* (86) and its difference in the sequence identity of the 5' domain (82). It was hoped that *ALS6* may exhibit adherence to fibroblasts or catheters because this may help to explore the substrate specificity of the *ALS6* and to identify the significance of the 5' domain in the adherence to fibroblasts and to catheters. Although the sequence identity of the 5' domain of *ALS2*, *ALS4*, *ALS7* and *ALS9* were

different from that of *ALS1*, *ALS3,* and *ALS5*, it is difficult to amplify them from genomic DNA of *Candida albicans* strain SC5314 (ATCC® number: MYA-2876) and sequencing of these genes was difficult to perform. As a result, these genes were not selected for the study.

5.6 Adhesion assay of transformed *Saccharomyces cerevisiae* to fibroblasts

The aim of this experiment was to study the adherence of *Saccharomyces cerevisiae* transformed with different *ALS* genes to fibroblasts. The adherence of *Saccharomyces cerevisiae* transformed with *ALS1* was highest, followed by *ALS6*, *ALS5*, and *Saccharomyces cerevisiae* transformed with empty plasmid. The result of this experiment also reconfirms the previous finding by Sheppard and associates (86).

In the experiment conducted by Sheppard and associates (86), *Saccharomyces cerevisiae* cells transformed with *ALS1* or *ALS5* were adhesive to human FaDu oral epithelial cells and to vascular endothelial cells. It is not surprising that *ALS1* and *ALS5* transformed *Saccharomyces cerevisiae* were adhesive to human fibroblasts. The result further demonstrated the wide substrate specificity of Als1 proteins and Als5 proteins.

Saccharomyces cerevisiae transformed with *ALS6* was shown by Sheppard and associates to be not adhesive to the epithelial cells and endothelial cells. Indeed, in that experiment, it only adhered to gelatin. Without obvious evidence suggesting

the result was an artefact, the high percentage of adherence to fibroblasts of *ALS6* transformed *Saccharomyces cerevisiae* is surprising. The adhesion of *ALS6* transformed *Saccharomyces cerevisiae* implicates that the ligands of other Als proteins may be very specific. Therefore, it may be worth to test the adherence of Als7, Als9 proteins, which were tested to exhibit no or low level of adherence to a number of substrata (86), to a wider range of cells and inanimate materials for the discovery of the binding partners of these proteins. The ability for *Candida albicans* to infect many different organs may be partially accounted for if each Als protein has a specific binding substrate. In addition, ligands of each Als proteins may be used for blocking the adhesion of *Candida albicans*.

An interesting observation is that the transformation of empty plasmid may increase the adherence of *Saccharomyces cerevisiae*. The percentage of adherence of *Sacchromyces cerevisiae* in the preliminary study of adherence to fibroblasts was 0.29% and that of *Saccharomyces cerevisiae* transformed with empty plasmid in this experiment was 10.35%. In addition, the percentage of adherence of all the four clones of *Saccharomyces cerevisiae* was higher than that of *Candida albicans* obtained in the preliminary study. Although the result cannot be compared directly as YPD broth was used for the culture of *Candida albicans* and of *Saccharomyces cerevisiae* in the preliminary study instead of using SC synthetic medium with

galactose, it is worth to further investigate whatever the difference was due to the medium used or the transformation of plasmid. If the difference was due to the use of different medium, it may be worth to investigate if there is any change in gene expression profile or in hydrophobicity of *Saccharomyces cerevisiae* cells. If the difference was due to the transformation, it may be worth to investigate if there is any change in the gene expression profile of *Saccharomyces cerevisiae* cells or if there is any element of the plasmid causing the difference.

5.7 Adhesion assay of transformed *Saccharomyces cerevisiae* to catheters

The aim of this experiment is to study the adherence to FEP, polyurethane, and silicone catheters of *Saccharomyces cerevisiae* transformed with different *ALS* genes. Transformation of *ALS1* or *ALS6* conferred *Saccharomyces cerevisiae* cells increased adherence to FEP catheters but not to polyurethane, or silicone catheters. Transformation of *ALS5* did not confer *Saccharomyces cerevisiae* adherence to all of the three catheters.

Both *ALS1* and *ALS6* transformed *Saccharomyces cerevisiae* had higher adherence to FEP catheters than control but not *ALS5* transformed *Saccharomyces cerevisiae*. It is worth to know why transformation of *ALS5* gene did not confer *Saccharomyces cerevisiae* adherence to FEP catheters. It should be noted that the 5' domain sequence of *ALS1* and *ALS5* shares 85% of identity. It was demonstrated by

Sheppard and associates that the N terminal of Als proteins is responsible for the adhesive function of the proteins by exchanging the 5' domain sequences of *ALS5* and of *ALS6*, resulting in exchange of adhesion profile (86). The same strategy can be used for elucidating which domain is responsible for the adherence to FEP catheters by exchanging the 5' domain sequence of *ALS1* and of *ALS5*. It may shed light on how Als proteins mediate adhesion to catheters. If the adhesive function to FEP catheters resides on the N terminal domain, it may be worth to find out if the adherence is due to the difference in the hypervariable regions of the N terminal domains (86) or factors such as the hydrophobicity of the Als proteins. It should be noted that the N terminal domain of Als proteins is hydrophobic.

Another interesting question is why *ALS1* and *ALS6* transformed *Saccharomyces cerevisiae* did not adhere to silicone catheters. It should be noted that the water contact angle of FEP and of silicone are 108° and 109° respectively (42). This implicates that the hydrophobicity of the two materials is similar. Although it should be noted that direct comparison of adherence to the two catheters is inappropriate due to the difference in the geometry and uncertain chemical composition of the two catheters, it may still be worthy to further investigate by using pure FEP and silicone disc as substrata. It may shed light on the role of hydrophobicity in the adhesion of Als proteins to catheters.

The hydrophilic nature of polyurethane (water contact angle is 78°) may explain why all *ALS* transformed *Saccharomyces cerevisiae* clones were not more adherent than *Saccharomyces cerevisiae* transformed with empty plasmid. The finding may also explain the clinical observation that the rate of infection associated with polyurethane catheters is lower than catheters using other materials (2). However, as the Als proteins from only one *Candida albicans* strain were tested, the result is not representative. It may be worth to screen other *Candida albicans* strains for a range of adherence to polyurethane catheters and transformed the *ALS* genes from those strains to *Saccharomyces cerevisiae* for adhesion assay.

5.8 Alternative research model

The studies of the adhesive functions of Als proteins mainly deploy either a gain of function study or a loss of function study. Gain of function is conducted by transforming a target *ALS* gene into *Saccharomyces cerevisiae*, which exhibits no adherence, and the adherence of the transformed *Saccharomyces cerevisiae* is measured. Loss of function study is conducted by knocking out the target *ALS* gene of *Candida albicans* using Ura blasting technique (88), and the adherence of the mutant is measured.

The gain of function study has the advantage of being easier to produce transformed *Saccharomyces cerevisiae*. However, a positive result (gain in adherence

by *Saccharomyces cerevisiae*) is less confirmative comparing with the loss of function study as the possibility of interactions between the protein products of the transformed genes and that of the gene from *Saccharomyces cerevisiae* could not be ruled out. The positive result (loss in adherence by *Candida albicans* mutant) of a loss of function study is very confirmative if the result is further confirmed by the restoration of adherence by reversing the mutant. However, a negative result in this kind of experiment may be the consequence of the compensatory functions exhibited by other adhesive proteins of the mutant. In addition, a double mutant of a same gene is difficult to obtain (88).

5.9 Implications and future work

It is the first time reported that transformation of *ALS* genes to *Saccharomyces cerevisiae* conferred its adherence to FEP catheters. This study shown that cell wall proteins of *Candida albicans* may contribute to the adhesion of *Candida albicans* cells to catheter surfaces and to fibroblasts. The results also shown that different Als proteins exhibited specific adhesion properties to catheters. In terms of clinical applications, the identification of cell structures responsible for adhesion of *Candida albicans* cells to catheter surface provided targets for preventing the adherence of *Candida albicans* cells to catheter surface, thus in turn, may reduce the incidence of CAI. By disrupting the cell wall proteins or other cell structure

responsible for adhesion, or by quenching the function of these structures, the adhesion of *Candida albicans* cells may be prevented. It was previously reported by Bjerketorp that adhesive proteins of *Staphylococcus aureus* were involved in the adhesion to CVC coated with serum proteins (109). The two findings together may imply that protein molecules of microbial cells may directly participate in adhesion to catheters and these proteins may exhibit different specificity or affinity to different catheter materials. Therefore, it may be worth to search for other proteins in different causative agents of CAI that may also exhibit adhesive function to catheters. By studying their adhesion properties to different catheter materials, an adhesion profile of different catheter materials can be constructed. The profiling may help to identify which catheter materials exhibit lowest affinity to the most common causative microorganisms of CAI and in turn, providing microbiological basis in the choice of materials for manufacturing catheters with low adherence to microbial cells.

In the aspect of science in adhesion, the exhibition of specific adhesion properties by Als1 protein and Als6 protein to FEP catheters and the exhibition of wide substrate specificity to human cells by Als1 protein and Als5 protein raised the question of what account for these differences. As the function of a molecule is largely determined by its structure, the specificity exhibited by these Als proteins is probably related to their structures. The differences in the structure may affect

whatever the proteins mediate adhesion through receptor binding, hydrophobicity or other mechanisms. And adhesion of Als proteins to cells and to catheters may be contributed by different mechanisms. As a result, characterization of the structure of these proteins is helpful in solving these questions. Indeed, understanding of the structure and function of these proteins is important in developing strategies for disrupting the structure or quenching the function of these proteins as the strategies for preventing adhesion due to receptor binding is very likely to be different from that due to hydrophobicity. This may ultimately decrease the incidence of CAI and disseminated candidiasis. Strategies may include altering the hydrophobicity of catheter surfaces or coating adhesion blockers on catheter surfaces.

The role of Als proteins in the pathogenesis of CAI, however, have to be further studied and confirmed as there are a lot of questions remain unanswered. These questions include whatever these proteins retain their adhesion properties in clinical situation, for example, under the shearing force during fluid administration in physiological conditions and under the effect drugs administrated; whatever these genes are expressed in *Candida albicans* cells adhered on catheters; whatever these proteins adhere to other catheter materials; whatever the Als proteins in other strains of *Candida albicans* also possess the same adhesion properties, and whatever these proteins are necessary and sufficient for initiating CAI. All these questions have to be

answered before the role of Als proteins in the pathogenesis of CAI can be firmly established.

For future work, first of all, it is necessary to confirm that the increased adherence of *ALS* transformed *Saccharomyces cerevisiae* to FEP catheters was due to the *ALS* genes instead of other factors associated with transformation. Gain of function study was used in this study. The result could be confirmed by loss of function study. It can be achieved by knocking out the *ALS* gene transformed in *Saccharomyces cerevisiae* and measuring its adherence. If the adherence of these *Saccharomyces cerevisiae* was the same as that of *Saccharomycese cerevisiae* transformed with empty plasmid, the increased adherence of *ALS* transformed *Saccharomyces cerevisiae* was due to the *ALS* genes. The result can also be confirmed by measuring the adherence of *Candida albicans* with the target *ALS* gene being knocked out.

Other future work may include studying the adherence of different Als proteins under shearing force in physiological conditions using a robbin device, studying the adherence of Als proteins of other clinical isolates of *Candida albicans*, investigate the *ALS* genes expression profile of *Candida albicans* adhered on catheter surfaces etc.

Sheppard and associates shown that the adhesive functions of Als proteins

to endothelial cells and to epithelial cells resides in the N terminal domain and proposed that N terminal domain is an immunoglobulin. This finding leads to the thinking that Als proteins mediate adhesion through receptor binding. It is worth to elucidate if the same N terminal domain is responsible for the adherence of Als proteins to FEP catheters. Another strategy for understanding the adhesion mechanism of Als proteins is to conduct ELISA to test the adherence of Als proteins to a number of different receptors. It may also be worth to investigate if the transformation of *ALS* genes results in a change of cell surface hydrophobicity using microbial adhesion to hydrocarbons (MATH) (34). If Als proteins confer adherence through receptor binding, it is worth to know what are the ligands for these Als proteins. It may be possible that Als proteins which possess a domain with similar molecular structure, bind to different ligands, different cells or catheters. This may explain the wide range of substrate specificity of Als proteins.

Chapter 6 Conclusion

In this study, it was found that adherence of *Candida albicans* to 0.5cm of FEP, polyurethane, and silicone catheters was not affected by initial inoculum size of *Candida albicans* cells in the range from $1x10^5$ cells to $1x10^7$ cells. The ATP bioluminescence used in this study was more sensitive than the safranin staining method. When the initial inoculum size was $1x10^7$ cells, the ATP bioluminescence method can detect 0.1% of adherence, which was sensitive enough for adhesion assays aim at studying initial adhesion events. *ALS1*, *ALS5* smaller allele, and *ALS6* contributed to the adherence of *Candida albicans* to human fibroblasts. *ALS1* and *ALS6* contributed to the adherence of *Candida albicans* only to FEP catheters. *ALS5* smaller allele did not contribute to the adherence of *Candida albicans* to the three catheters tested.

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Table 1. Turbidity of YPD broth in the preliminary studies of adherence of *Candida albicans* to FEP, polyurethane, and silicone catheter fragments. Turbid YPD culture was marked as '+' and clear YPD culture was marked as '-'.

Catheters	FEP	Polyurethane	Silicone
Experimental Group	+	+	+
Control Group	-	-	-

Table 2. Nucleotide mutations and consequent amino acid changes in the *ALS1*, *ALS5* smaller allele, and *ALS6* inserted into plasmids.

Gene	Position (bp)	Nucleotide change	Amino acid change
ALS1	1933	C → T	Leu → Phe
***ALS5* smaller allele**	48	T → C	Synonymous
***ALS5* smaller allele**	258	G → A	Synonymous
ALS6	3529	A → G	Thr → Ala

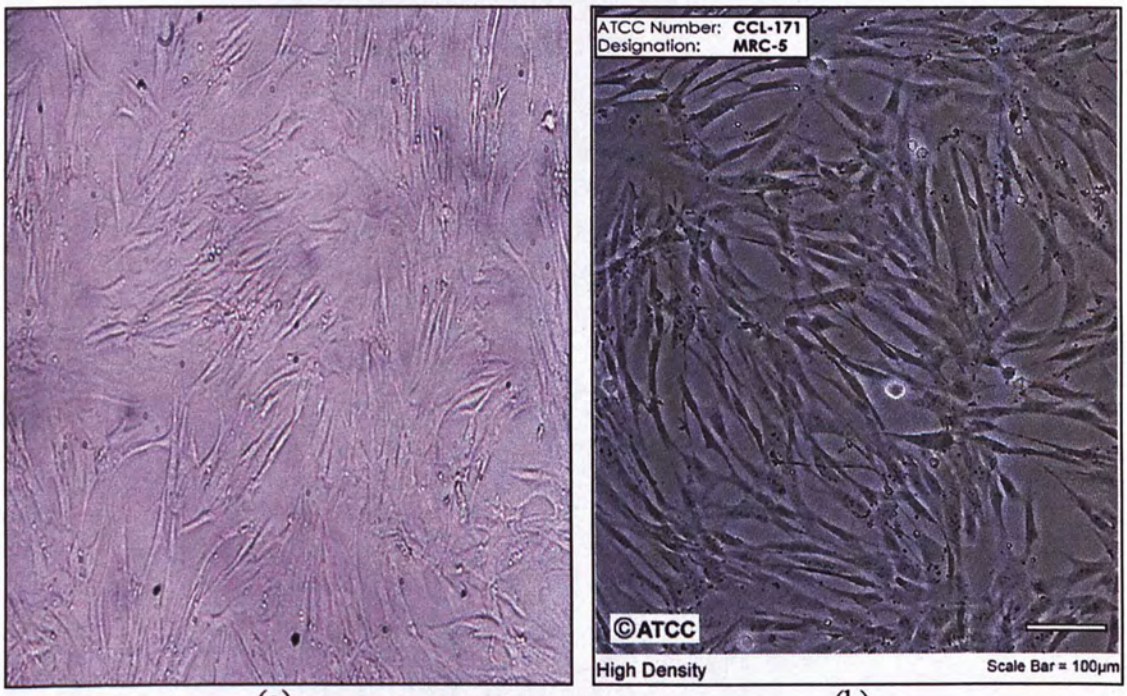
Table 3. The percentage of initial inoculum adhered to fibroblasts of *Saccharomyces cerevisiae* transformed with empty plasmid, *ALS1*, *ALS5* smaller allele, and *ALS6*.

Clone	Control	*ALS1*	*ALS5* smaller allele	*ALS6*
Percentage of initial inoculum adhered to fibroblasts	10.35897	44.86979	24.01747	27.69231

Table 4. The percentage of initial inoculum of *Saccharomyces cerevisiae* transformed with empty plasmid, *ALS1*, *ALS5* smaller allele, and *ALS6* adhered to FEP, polyurethane, and silicone catheter fragments. The adherence of *ALS6* transformed *Saccharomyces cerevisiae* to polyurethane, and silicone catheters was below the detection limit of the assay and was marked as 'N/A'.

Catheter	Control	*ALS1*	*ALS5* smaller allele	*ALS6*
FEP	0.168706	3.934466	0.370759	1.017344
Polyurethane	0.158943	0.282257	0.286522	N/A
Silicone	0.471769	2.568740	0.192258	N/A

Figure 1. Morphology and confluence of fibroblasts. (a) Morphology of fibroblasts culture after thawing; (b) Morphology of fibroblasts (CCL-171) from ATCC.



(a) (b)

Figure 2. A monolayer of fibroblasts of 100% confluence in a well of a 6-well culture plate inoculated with $2x10^6$ cells after 24 hours of incubation.

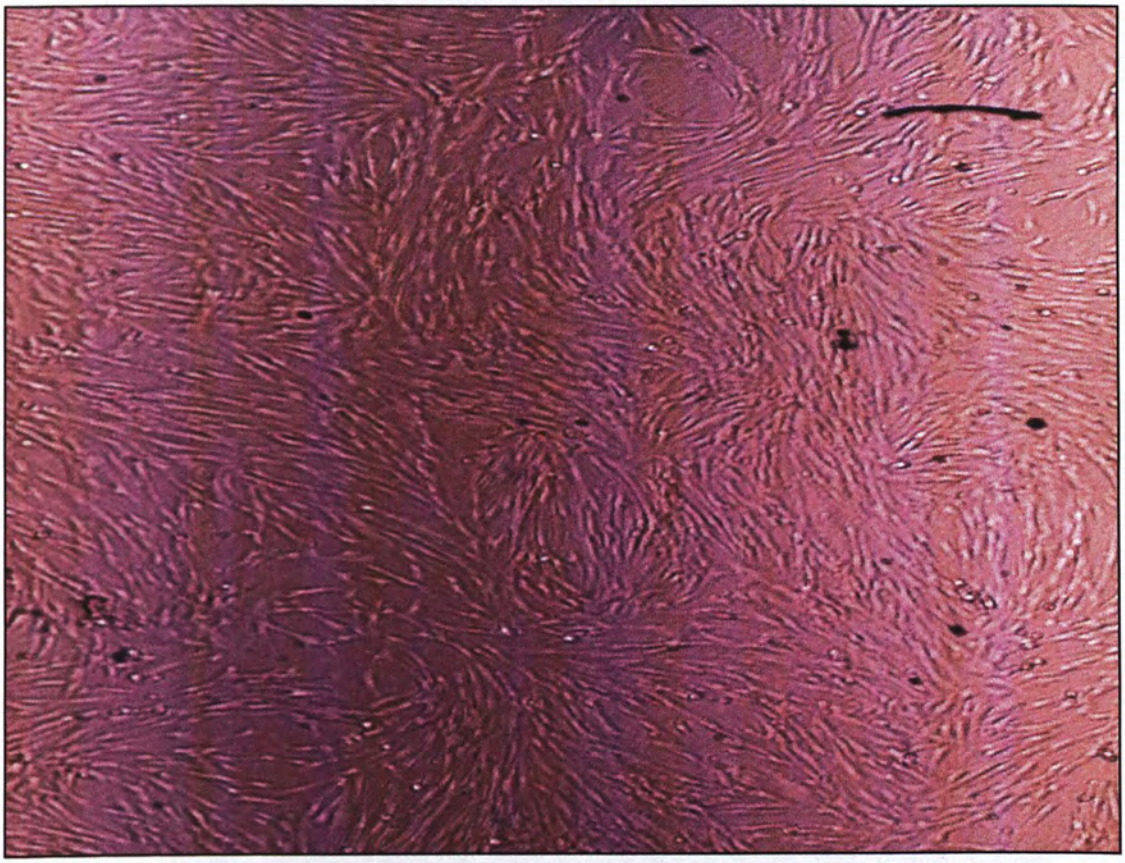


Figure 3. The percentage of initial inoculum adhered to fibroblasts in the preliminary adhesion assay of *Candida albicans*.

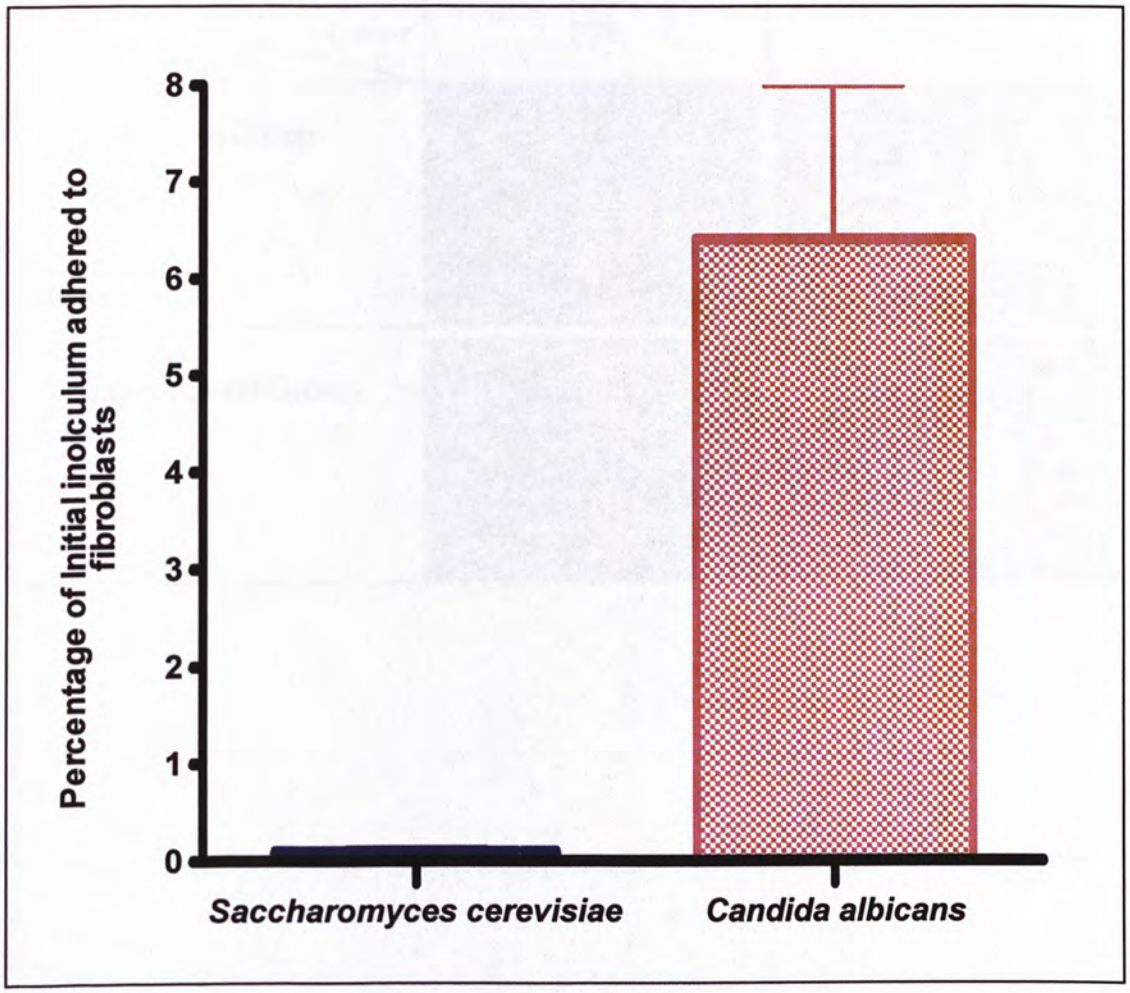


Figure 4. Surface of FEP catheter fragments and of polyurethane catheter fragments after adhesion of *Candida albicans*.

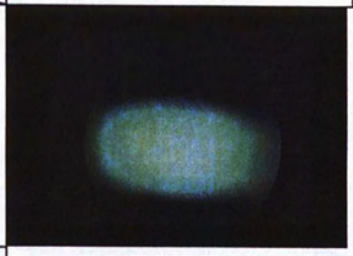
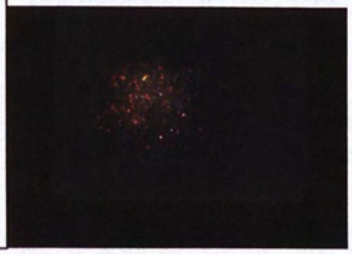
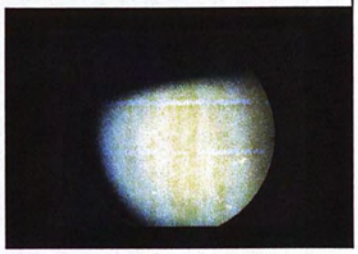
Catheters	FEP	Polyurethane
Control Group		
Experimental Group		

Figure 5. RT-PCR of *ALS1*, *ALS5* smaller allele, and *ALS6* of *Candida albicans* cultured in YPD broth. Lane M: 1kb trackit molecular weight marker; Lane 1: negative control of RT-PCR of *ALS1*; Lane 2: negative control of RT-PCR of *ALS5* smaller allele; Lane 3: negative control of RT-PCR of *ALS6*; Lane 4: PCR control of *ALS1* using RNA as template; Lane 5: PCR control of *ALS5* smaller allele using RNA as template; Lane 6: PCR control of *ALS6* using RNA as template; Lane 7: RT-PCR of *ALS1*; Lane 8: RT-PCR of *ALS5* smaller allele; Lane 9: RT-PCR of *ALS6*.

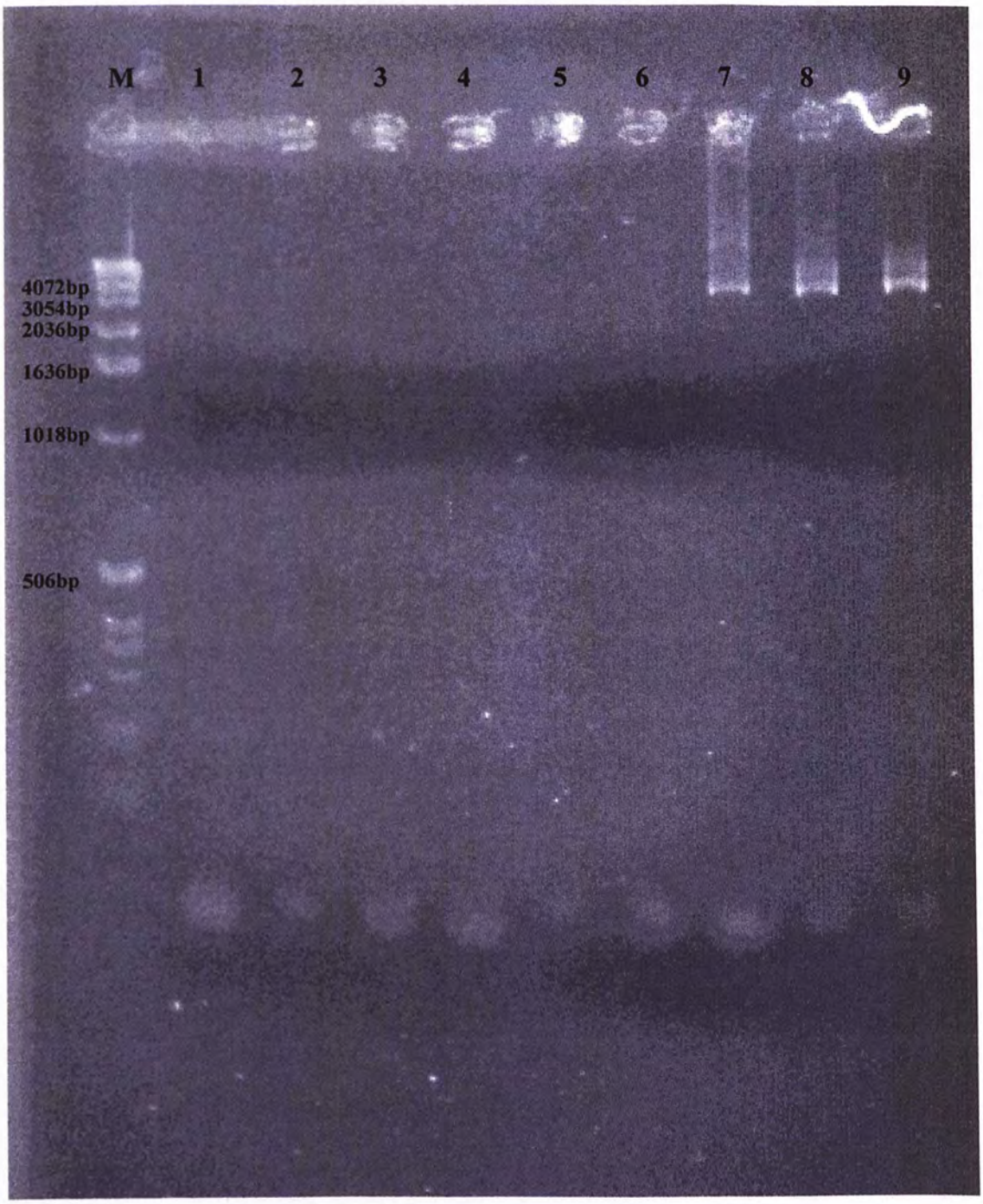


Figure 6. Quantitation system of adhesion assay using absorbance measurement of *Candida albicans* stained with safranin.

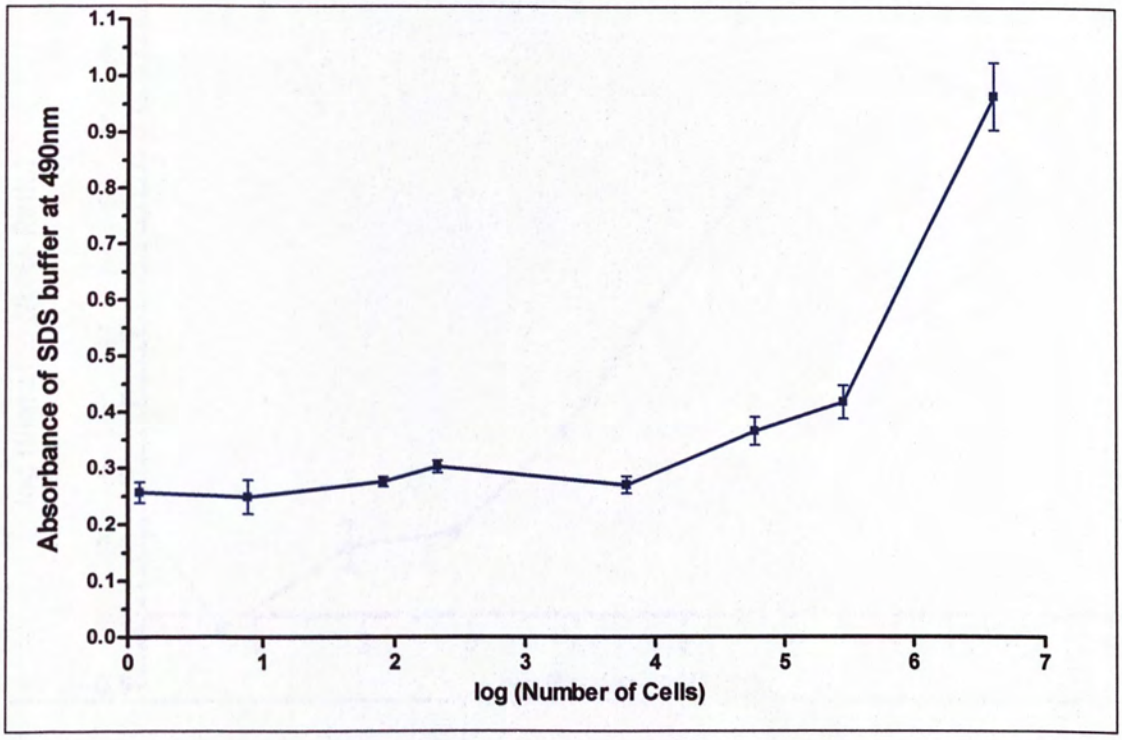


Figure 7. Quantitation system of adhesion assay using ATP bioluminescence.

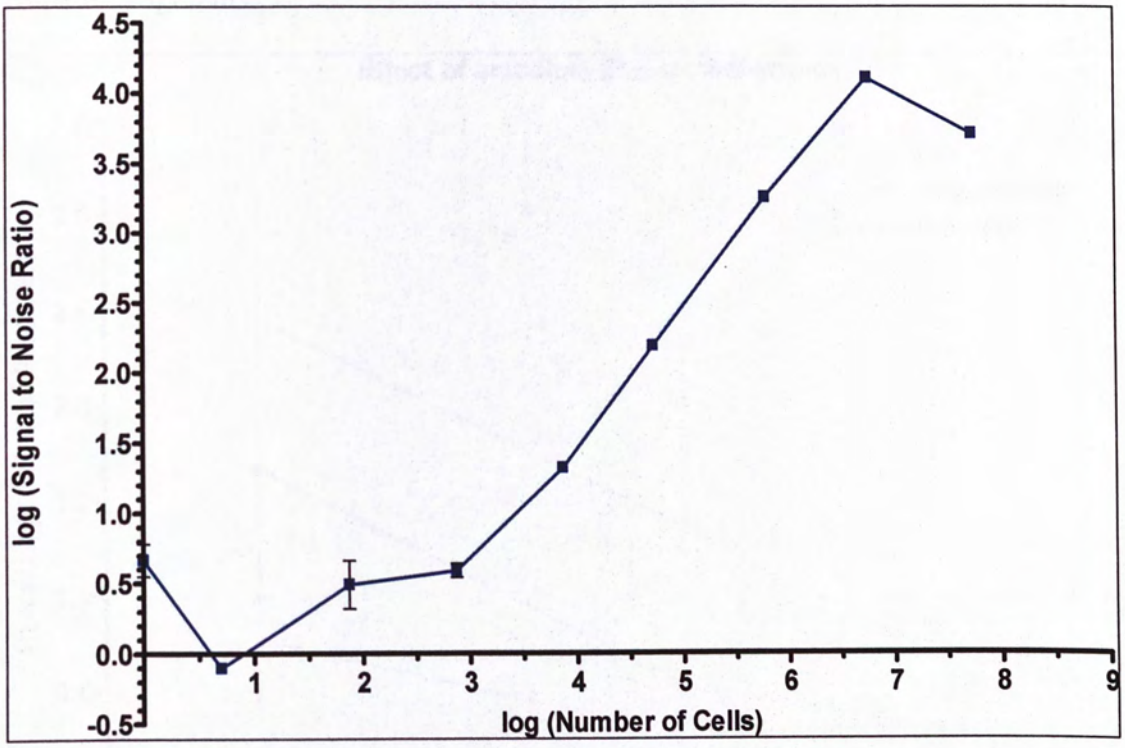


Figure 8. Effect of inoculum size on adhesion to FEP, polyurethane, and silicone catheter fragments.

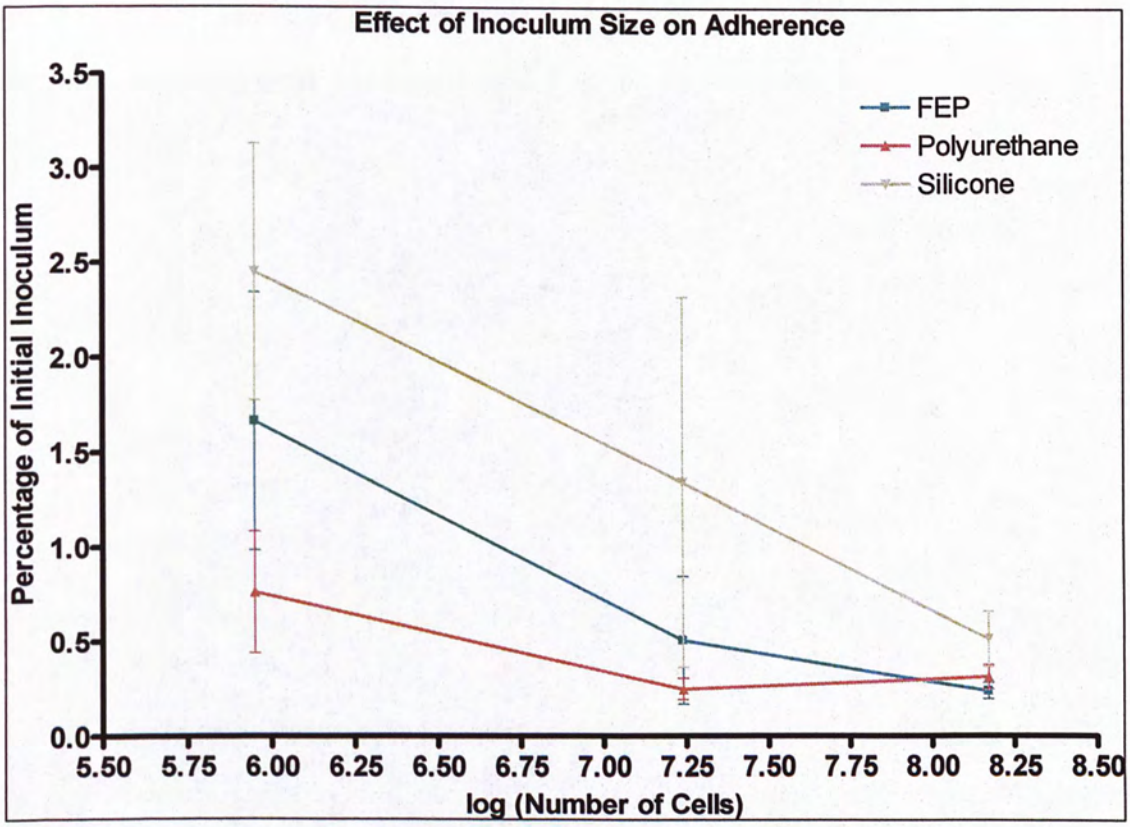


Figure 9. PCR of *ALS1*, *ALS5* smaller allele, and *ALS6* of *Candida albicans*. Lane M: 1kb trackit molecular weight marker; Lane 1: negative control of *ALS1*; Lane 2: negative control of *ALS5* smaller allele; Lane 3: negative control of *ALS6*; Lane 4: PCR of *ALS1*; Lane 5: PCR of *ALS5* smaller allele; Lane 6: PCR of *ALS6*.

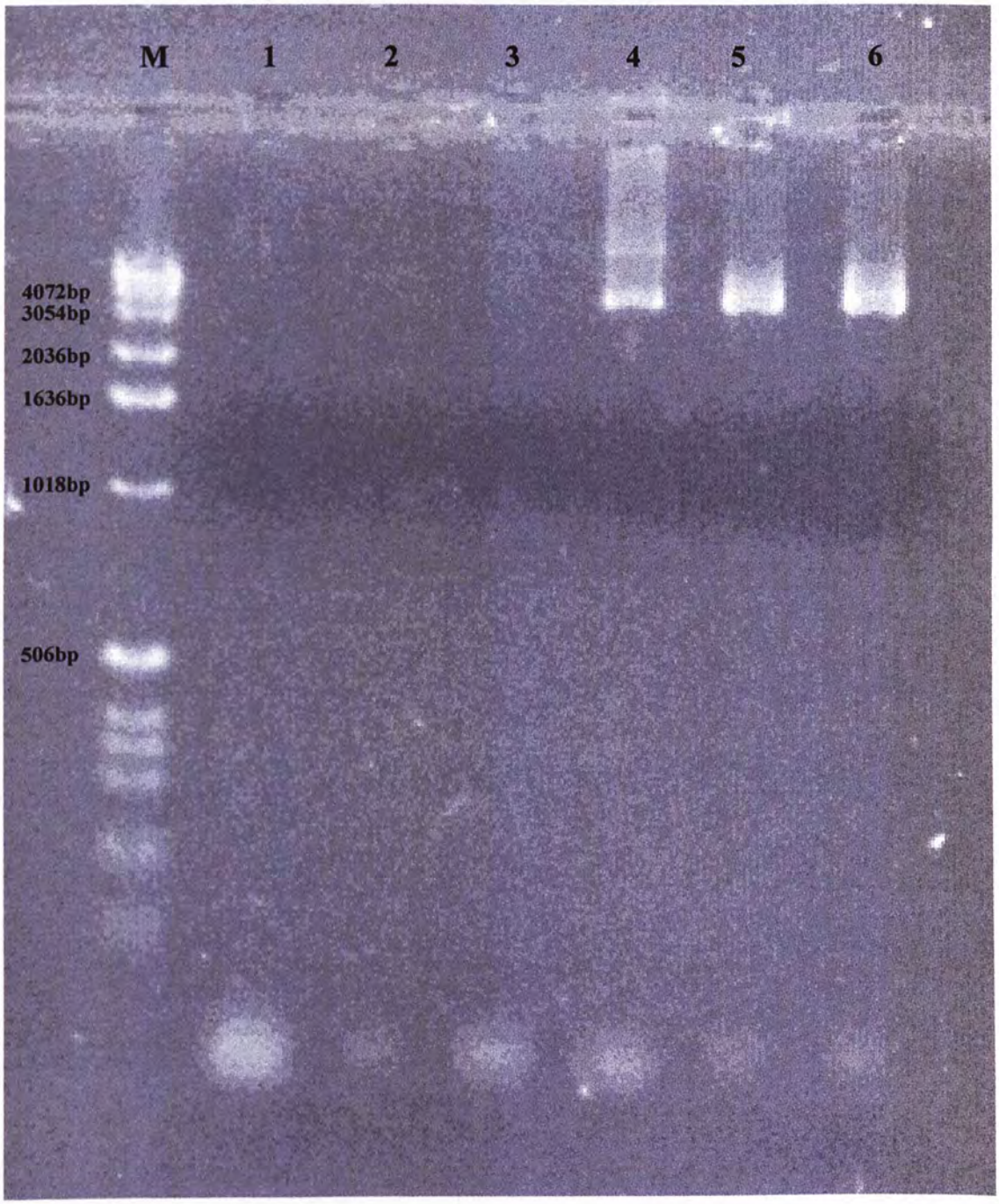


Figure 10. Confirmation of ligation of *ALS* genes by PCR. Lane M: 1kb trackit molecular weight marker; Lane 1: negative control of PCR of *ALS1*; Lane 2: negative control of PCR of *ALS5* smaller allele; Lane 3: negative control of PCR of *ALS6*; Lane 4: PCR of empty plasmid using *ALS1* primers; Lane 5: PCR of empty plasmid using *ALS5* primers; Lane 6: PCR of empty plasmid using *ALS6* primers; Lane 7: PCR product of *ALS1* from DNA of *Candida albicans*; Lane 8: PCR product of *ALS5* smaller allele from DNA of *Candida albicans*; Lane 9: PCR product of *ALS6* from DNA of *Candida albicans*; Lane 10: PCR of *ALS1* from *ALS1* ligated plasmid; Lane 11: PCR of *ALS5* smaller allele from *ALS5* smaller allele ligated plasmid; Lane 12: PCR of *ALS6* from *ALS6* ligated plasmid.

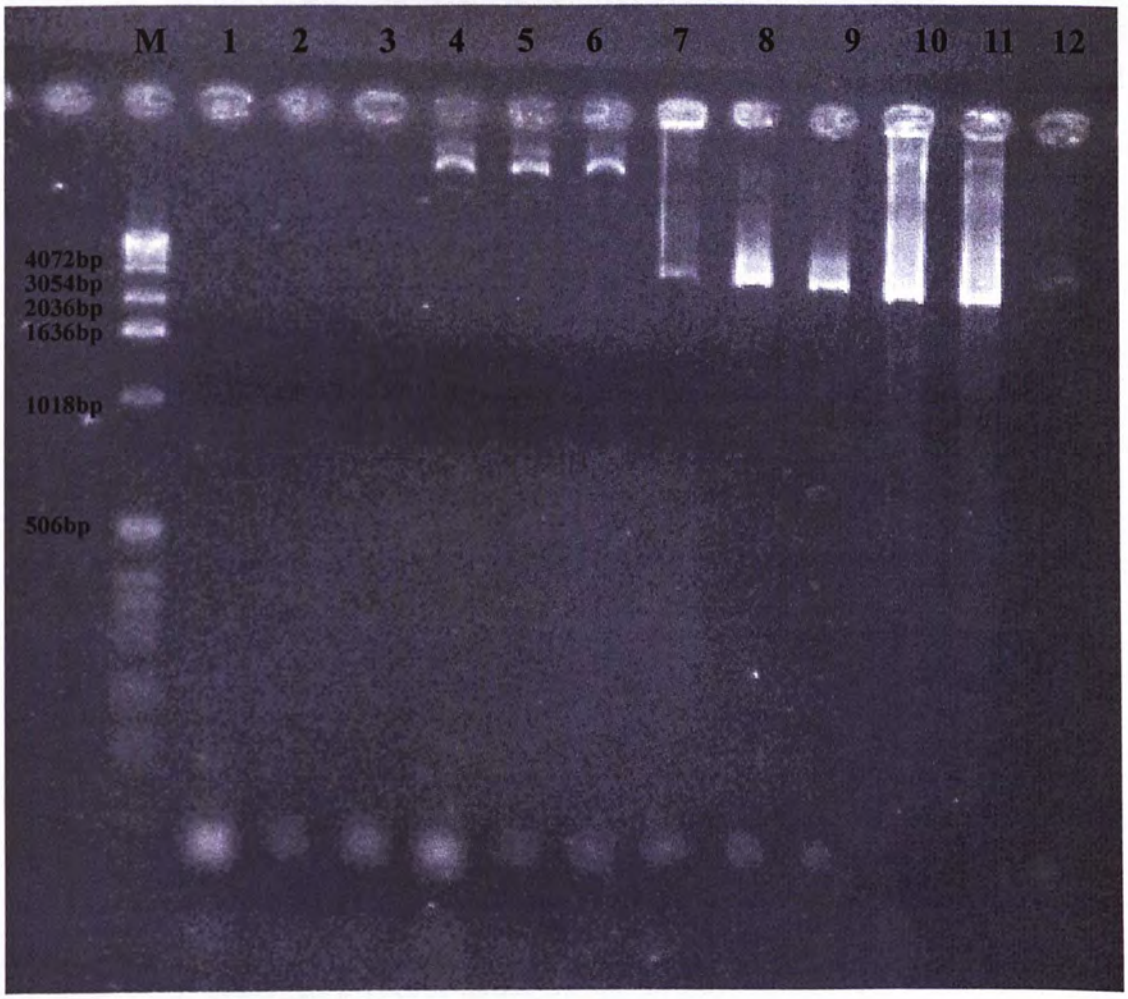


Figure 11. Dot blot of cell wall lysates of *Saccharomyces cerevisiae* transformed with empty plasmid, *ALS1*, *ALS5* smaller allele, and *ALS6*.

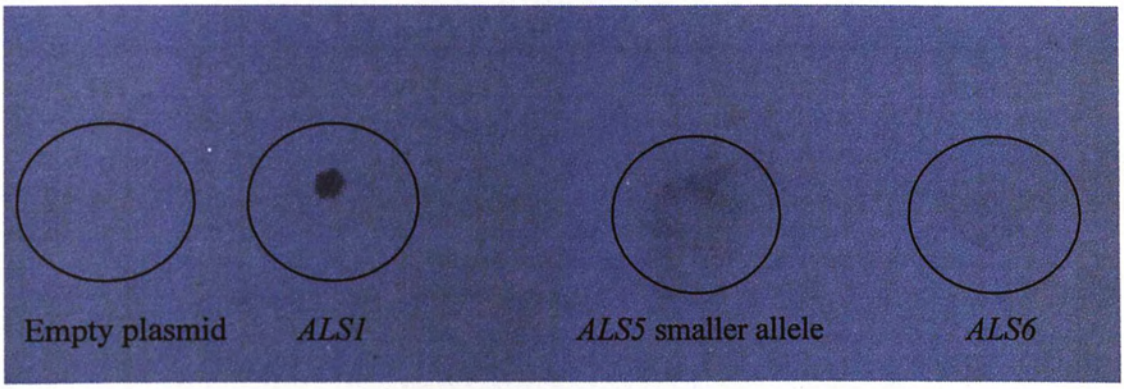


Figure 12. The percentage of initial inoculum of *Saccharomyces cerevisiae* transformed with empty plasmid, *ALS1*, *ALS5* smaller allele, and *ALS6* adhered to fibroblasts.

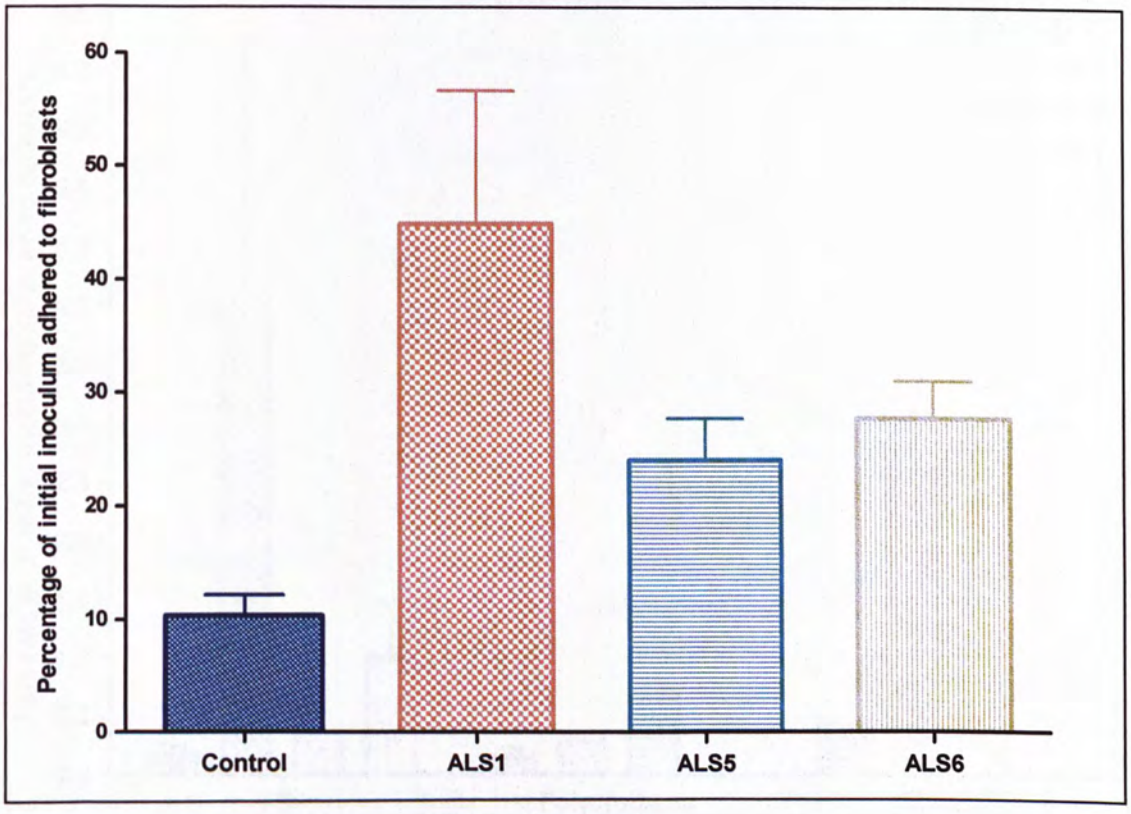
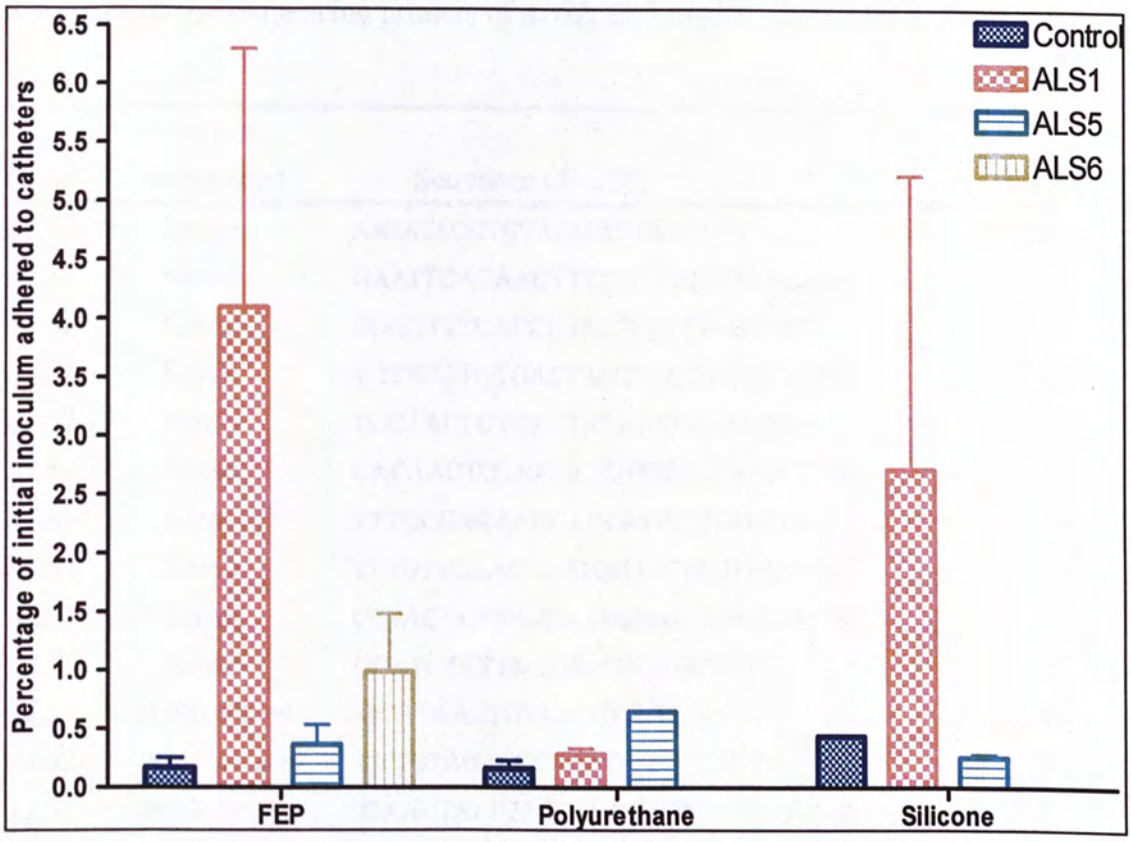


Figure 13. The percentage of initial inoculum of *Saccharomyces cerevisiae* transformed with empty plasmid, *ALS1, ALS5* smaller allele, and *ALS6* adhered to FEP, polyurethane and silicone catheters.



Appendix I

Sequence of the sequencing primers of *ALS1*, *AL5* smaller allele, and *ALS6*.

Gene	Strand Sequenced	Sequence (5' – 3')	Tm(℃)
ALS1	Sense	AATATACCTCTATACTTTAACGTC	50
ALS1	Sense	GAATTCACAACTTTTTCTACATTAACATG	51
ALS1	Sense	CCGGTTTCATCTGAATCATTTAGTTAC	53
ALS1	Sense	TGTTGGTGTGACTACTTCCTATCTGACTA	53
ALS1	Sense	TCCAACTGTCACTACAACCGAGTATT	52
ALS1	Sense	CACAACTGTAACTGGTCCACCAAGT	53
ALS1	Sense	TTTGCCACAACCACCACAGTTACT	54
ALS1	Sense	TTTCTTCAACTGATGGTATTAGTGCTACAT	54
ALS1	Sense	CCTACAGGTGATAATGGAGACAATACTTC	53
ALS1	Sense	GGATCTGTTACTGGTGGAGCTGTT	52
ALS1	Anti- Sense	GCGTGAATGTAAGCGTGAC	50
ALS1	Anti- Sense	CTTGTAGATGCTGAGGTGCCTGTT	53
ALS1	Anti- Sense	GAAGTATTGTCTCCATTATCACCTGTAGG	53
ALS1	Anti- Sense	TAGCACTAATACCATCAGTTGAAGAAAGT	52
ALS1	Anti- Sense	ACTGTGGTGGTTGTGGCAAATGAT	56
ALS1	Anti- Sense	ATAACAGTATCAGTGCCACTTGGTG	51
ALS1	Anti- Sense	AATACTCGGTTGTAGTGACAGTTGGAT	53
ALS1	Anti- Sense	AGTCAGATAGGAAGTAGTCACACCAACATA	53
ALS1	Anti- Sense	ACCGGATAATTCCAATCATTTAATC	51
ALS1	Anti- Sense	CATGTTAATGTAGAAAAAGTTGTGAATTC	51
ALS5	Sense	AATATACCTCTATACTTTAACGTC	50
ALS5	Sense	AATGTTTTACTGCTGGTACCAATACGGT	56
ALS5	Sense	TAAGAATAGTGATGCCGGATCTAAC	50
ALS5	Sense	AATTGACACAGTGGTGGTACAAGTTC	52
ALS5	Sense	TATCGTTATACATGATCCATTGGAAG	50
ALS5	Sense	GTTATTACCCATCTAGCACAATTCTG	50
ALS5	Sense	TTGTAACAGGGATACCATCGTCTAGT	51
ALS5	Sense	CAACTCCGTCAAGTCATTCTGAAATA	53
ALS5	Sense	CCAAGTAAAACTAAGTCGATTGAAGA	50
ALS5	Anti- Sense	GCGTGAATGTAAGCGTGAC	50

ALS5	Anti- Sense	GTGGAGTACTGGCAACATTGCTAAA	53
ALS5	Anti- Sense	GTTGAAGAACTAGCACTTGTTTGCAG	53
ALS5	Anti- Sense	GCTTGTTGGGTCACTTGTAAGAGAGA	54
ALS5	Anti- Sense	TGGCATGTCTGAACTTAATGGTATAT	50
ALS5	Anti- Sense	CAGTTCTTCCAATGGATCATGTATAA	50
ALS5	Anti- Sense	CTGGGTTGTAGTTGTAGTTGGATTTG	52
ALS5	Anti- Sense	ATAAATGGACGATAACCGGCAGGAAC	58
ALS5	Anti- Sense	ATGATCTCAAATTATTGTTCACTGTACAT	50
ALS6	Sense	AATATACGACTCCTATAGGA	55
ALS6	Sense	TCAGGCTACCAATTTCATTCAATGTG	55
ALS6	Sense	ATTGTATGGATGGCAAAAAGGGAAAT	56
ALS6	Sense	AACGGATAGTGTGATCATCAAGGAGCCACACAAC	54
ALS6	Sense	CAATTGGGTTAACATCTAGTGAGTCG	52
ALS6	Sense	GATTTCTAGTGATTCGGTGTCTCTTAC	50
ALS6	Sense	ATCCAAATATCGACTCACTGCAAACA	55
ALS6	Sense	ACAGTATCATCTGTATCAGTACATTCAATAGA	59
ALS6	Sense	CCAAATTCCTTGATTCATTCAGAGTC	53
ALS6	Anti- Sense	GCGTGAATGTAAGCG	55
ALS6	Anti- Sense	GCTGCCGACGACTTATCATCAA	53
ALS6	Anti- Sense	AAAGTGTTTGGTTCGTTATCTTCGAT	53
ALS6	Anti- Sense	ATCTCCGTTATCACGACGACAAAA	53
ALS6	Anti- Sense	TAGACCTTGCATCACTTGAAACTAAAG	52
ALS6	Anti- Sense	GACAGTTCTTCCAATGGATCATGTATA	52
ALS6	Anti- Sense	ACTTGATGTGGTAGCTGATCCAGTCC	55
ALS6	Anti- Sense	GAAAATGATTCTGATGATACTGGCAT	52
ALS6	Anti- Sense	TACTGAAGTTTGTGTGGTAATAAATTTGAAAA	55

Appendix II

Sequencing result of *ALS1* inserted in plasmid.

Appendix II (cont'd)

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          ****************************************************************************************************
Reference TTACAGACAGTACCACTGCTGTCACTACTTTACCATTCAATCCAAGTGTTGATAAAACCAAAACAATCGAAATTTTGCAACCTATTCCAACCACTACCAT  1100
   Result TTACAGACAGTACCACTGCTGTCACTACTTTACCATTCAATCCAAGTGTTGATAAAACCAAAACAATCGAAATTTTGCAACCTATTCCAACCACTACCAT  1100

          ****************************************************************************************************
Reference CACAACTTCATATGTTGGTGTGACTACTTCCTATCTGACTAAGACTGCACCAATTGGTGAAACAGCTACTGTTATTGTTGATGTGCCATATCATACTACC  1200
   Result CACAACTTCATATGTTGGTGTGACTACTTCCTATCTGACTAAGACTGCACCAATTGGTGAAACAGCTACTGTTATTGTTGATGTGCCATATCATACTACC  1200

          ****************************************************************************************************
Reference ACAACTGTTACCAGTGAATGGACAGGAACAATCACTACCACTACAACTCGTACCAATCCAACTGATTCAATTGACACAGTGGTGGTACAAGTTCCACTGC  1300
   Result ACAACTGTTACCAGTGAATGGACAGGAACAATCACTACCACTACAACTCGTACCAATCCAACTGATTCAATTGACACAGTGGTGGTACAAGTTCCACTGC  1300

          ****************************************************************************************************
Reference CAAATCCAACTGTTAGTACTACTGAATATTGGTCTCAGTCCTATGCTACAACCACTACAGTTACTGCTCCTCCAGGTGGTACCGATACTGTTATCATTAG  1400
   Result CAAATCCAACTGTTAGTACTACTGAATATTGGTCTCAGTCCTATGCTACAACCACTACAGTTACTGCTCCTCCAGGTGGTACCGATACTGTTATCATTAG  1400

          ****************************************************************************************************
Reference AGAGCCACCAAACCACACTGTTACTACTACTGAATATTGGTCACAATCATTTGCCACCACTACCACTGTAACTGCACCACCAGGTGAAACTGATACCGTT  1500
   Result AGAGCCACCAAACCACACTGTTACTACTACTGAATATTGGTCACAATCATTTGCCACCACTACCACTGTAACTGCACCACCAGGTGAAACTGATACCGTT  1500

          ****************************************************************************************************
Reference ATCATTAGAGAACCACCAAATCACACTGTTACTACTACTGAATACTGGTCACAATCCTATGCCACCACTACCACTGTTACTGCTCCTCCAGGTGGTACCG  1600
   Result ATCATTAGAGAACCACCAAATCACACTGTTACTACTACTGAATACTGGTCACAATCCTATGCCACCACTACCACTGTTACTGCTCCTCCAGGTGGTACCG  1600

          ****************************************************************************************************
Reference ATACCGTTCTTATCAGAGAGCCACCAAACCATACTGTTACTACTACTGAATACTGGTCACAATCCTATGCCACCACTACCACTGTAACTGCACCACCAGG  1700
   Result ATACCGTTCTTATCAGAGAGCCACCAAACCATACTGTTACTACTACTGAATACTGGTCACAATCCTATGCCACCACTACCACTGTAACTGCACCACCAGG  1700

          ****************************************************************************************************
Reference TGGTACTGACACTGTTATCATTAGAGAGCCACCAAACTACACGGTTACTACTACTGAATATTGGTCTCAATCATATGCAACCACTACTACCATTACCGCT  1800
   Result TGGTACTGACACTGTTATCATTAGAGAGCCACCAAACTACACGGTTACTACTACTGAATATTGGTCTCAATCATATGCAACCACTACTACCATTACCGCT  1800

          ****************************************************************************************************
Reference CCACCTGGTGAAACCGATACCGTTATCATTAGAGAACCACCAAATCACACTGTTACTACTACTGAATACTGGTCACAATCCTATGCCACCACTACCACTG  1900
   Result CCACCTGGTGAAACCGATACCGTTATCATTAGAGAACCACCAAATCACACTGTTACTACTACTGAATACTGGTCACAATCCTATGCCACCACTACCACTG  1900

          ******************************** *******************************************************************
Reference TTACTGCTCCTCCAGGTGGTACCGATACCGTTCTTATCAGAGAGCCACCAAACCACACTGTCACTACTACTGAATACTGGTCTCAATCATATGCTACAAC  2000
   Result TTACTGCTCCTCCAGGTGGTACCGATACCGTTTTTATCAGAGAGCCACCAAACCACACTGTCACTACTACTGAATACTGGTCTCAATCATATGCTACAAC  2000
```

Appendix II (cont'd)

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          ****************************************************************************************************
Reference CACCACTGTTACTGCACCACCAGGTGGTACCGATACCGTTCTTATCAGAGAGCCACCAAACCATACTGTTACTACTACTGAATACTGGTCACAATCCTAT  2100
   Result CACCACTGTTACTGCACCACCAGGTGGTACCGATACCGTTCTTATCAGAGAGCCACCAAACCATACTGTTACTACTACTGAATACTGGTCACAATCCTAT  2100

          ****************************************************************************************************
Reference GCCACCACTACCACTGTAACTGCACCACCAGGAGGTACCGATACTGTTATCATTAGAGAACCACCAAATCACACTGTTACTACTACTGAATACTGGTCAC  2200
   Result GCCACCACTACCACTGTAACTGCACCACCAGGAGGTACCGATACTGTTATCATTAGAGAACCACCAAATCACACTGTTACTACTACTGAATACTGGTCAC  2200

          ****************************************************************************************************
Reference AATCCTATGCCACCACTACCACTGTTACTGCTCCTCCAGGTGGTACTGACACTGTTATCATTAGAGAACCACCAAACTACACGGTTACTACTACTGAATA  2300
   Result AATCCTATGCCACCACTACCACTGTTACTGCTCCTCCAGGTGGTACTGACACTGTTATCATTAGAGAACCACCAAACTACACGGTTACTACTACTGAATA  2300

          ****************************************************************************************************
Reference TTGGTCACAATCATTTGCCACAACCACCACAGTTACTGCTCCTCCAGGTGGTACTGACACTGTGATTATCTATGAAAGCATGTCAAGTTCAAAGATTTCT  2400
   Result TTGGTCACAATCATTTGCCACAACCACCACAGTTACTGCTCCTCCAGGTGGTACTGACACTGTGATTATCTATGAAAGCATGTCAAGTTCAAAGATTTCT  2400

          ****************************************************************************************************
Reference ACATCCTCCAATGATATAACCAGTATCATTCCATCATTTTCCCGTCCTCATTATGTCAACAGCACAACCTCCGATTTGTCAACATTTGAATCTTCATCCA  2500
   Result ACATCCTCCAATGATATAACCAGTATCATTCCATCATTTTCCCGTCCTCATTATGTCAACAGCACAACCTCCGATTTGTCAACATTTGAATCTTCATCCA  2500

          ****************************************************************************************************
Reference TGAATACTCCTACTTCTATCAGTAGTGATGGTATGTTGTTGTCTTCTACAACTTTGGTTACTGAATCAGAAACAACTACAGAACTGATTTGCAGTGATGG  2600
   Result TGAATACTCCTACTTCTATCAGTAGTGATGGTATGTTGTTGTCTTCTACAACTTTGGTTACTGAATCAGAAACAACTACAGAACTGATTTGCAGTGATGG  2600

          ****************************************************************************************************
Reference TAAAGAGTGTTCTAGATTGTCCAGTTCTTCTGGTATTGTCACAAATCCAGATAGCAATGAATCCTCAATCGTAACTAGTACTGTTCCTACTGCAAGTACA  2700
   Result TAAAGAGTGTTCTAGATTGTCCAGTTCTTCTGGTATTGTCACAAATCCAGATAGCAATGAATCCTCAATCGTAACTAGTACTGTTCCTACTGCAAGTACA  2700

          ****************************************************************************************************
Reference ATGTCTGATTCACTTTCTTCAACTGATGGTATTAGTGCTACATCTTCTGATAATGTTTCAAAATCAGGAGTATCAGTTACAACCGAAACTTCTGTTACTA  2800
   Result ATGTCTGATTCACTTTCTTCAACTGATGGTATTAGTGCTACATCTTCTGATAATGTTTCAAAATCAGGAGTATCAGTTACAACCGAAACTTCTGTTACTA  2800

          ****************************************************************************************************
Reference CTATTCAAACTACTCCAAACCCATTATCATCTTCAGTGACATCATTGACTCAGTTGTCTTCAATTCCAAGTGTTTCAGAAAGTGAAAGTAAAGTTACATT  2900
   Result CTATTCAAACTACTCCAAACCCATTATCATCTTCAGTGACATCATTGACTCAGTTGTCTTCAATTCCAAGTGTTTCAGAAAGTGAAAGTAAAGTTACATT  2900

          ****************************************************************************************************
Reference TACAAGCAATGGAGACAACCAAAGTGGTACTCATGATTCACAATCTACTTCCACTGAAATTGAAATTGTAACAACCAGTTCTACTAAAGTTTTACCACCT  3000
   Result TACAAGCAATGGAGACAACCAAAGTGGTACTCATGATTCACAATCTACTTCCACTGAAATTGAAATTGTAACAACCAGTTCTACTAAAGTTTTACCACCT  3000
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Appendix II (cont'd)

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          ****************************************************************************************************
Reference GTCGTTTCTTCTAATACTGATTTGACTAGTGAACCAACAAATACCAGAGAACAACCAACTACATTATCAACTACTTCAAACTCCATCACTGAAGATATCA  3100
   Result GTCGTTTCTTCTAATACTGATTTGACTAGTGAACCAACAAATACCAGAGAACAACCAACTACATTATCAACTACTTCAAACTCCATCACTGAAGATATCA  3100

          ****************************************************************************************************
Reference CCACATCTCAACCTACAGGTGATAATGGAGACAATACTTCATCAACCAATCCAGTTCCAACTGTGGCAACAAGTACTTTAGCATCTGCAAGTGAAGAAGA  3200
   Result CCACATCTCAACCTACAGGTGATAATGGAGACAATACTTCATCAACCAATCCAGTTCCAACTGTGGCAACAAGTACTTTAGCATCTGCAAGTGAAGAAGA  3200

          ****************************************************************************************************
Reference CAACAAAAGCGGTTCTCATGAATCAGCATCCACAAGTTTGAAACCAAGTATGGGTGAAAATTCTGGATTAACTACTTCTACTGAAATTGAAGCTACAACA  3300
   Result CAACAAAAGCGGTTCTCATGAATCAGCATCCACAAGTTTGAAACCAAGTATGGGTGAAAATTCTGGATTAACTACTTCTACTGAAATTGAAGCTACAACA  3300

          ****************************************************************************************************
Reference ACCAGTCCTACAGAAGCTCCATCACCTGCTGTTTCTTCTGGTACTGATGTAACTACTGAACCAACTGATACTAGAGAACAACCTACTACATTATCAACTA  3400
   Result ACCAGTCCTACAGAAGCTCCATCACCTGCTGTTTCTTCTGGTACTGATGTAACTACTGAACCAACTGATACTAGAGAACAACCTACTACATTATCAACTA  3400

          ****************************************************************************************************
Reference CTTCAAAAACAAACAGTGAACTGGTTGCTACTACACAAGCTACTAATGAAAATGGTGGTAAATCTCCATCAACTGATTTAACATCAAGCTTGACAACAGG  3500
   Result CTTCAAAAACAAACAGTGAACTGGTTGCTACTACACAAGCTACTAATGAAAATGGTGGTAAATCTCCATCAACTGATTTAACATCAAGCTTGACAACAGG  3500

          ****************************************************************************************************
Reference CACCTCAGCATCTACAAGTGCTAATAGCGAACTTGTTACTAGTGGATCTGTTACTGGTGGAGCTGTTGCCAGTGCTTCAAATGATCAATCACATTCTACT  3600
   Result CACCTCAGCATCTACAAGTGCTAATAGCGAACTTGTTACTAGTGGATCTGTTACTGGTGGAGCTGTTGCCAGTGCTTCAAATGATCAATCACATTCTACT  3600

          ****************************************************************************************************
Reference TCTGTTACCAACAGCAACAGCATTGTATCTAATACCCCACAAACTACATTGAGTCAACAAGTTACCTCATCCTCACCTTCAACCAACACATTCATTGCTT  3700
   Result TCTGTTACCAACAGCAACAGCATTGTATCTAATACCCCACAAACTACATTGAGTCAACAAGTTACCTCATCCTCACCTTCAACCAACACATTCATTGCTT  3700

          ***********************************************************************************
Reference CTACATACGATGGCTCTGGTTCTATTATCCAACATTCTACTTGGTTGTACGGTTTGATCACATTATTGTCCTTGTTCATTTAG  3783
   Result CTACATACGATGGCTCTGGTTCTATTATCCAACATTCTACTTGGTTGTACGGTTTGATCACATTATTGTCCTTGTTCATTTAG  3783
```

Appendix III

Sequencing result of *ALS5* smaller allele inserted in plasmid.

Appendix III (cont'd)

Appendix III (cont'd)

Appendix III (cont'd)

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          ****************************************************************************************************
Reference GATACTGGTAATATTAATGCTGGGTCGCTGCACACAAGTACTGCTTCCATCAAAGAACTGTCAATTCAGAAAACTGGAGTAACGTTAAGTTCCAGTTATT  3100
   Result GATACTGGTAATATTAATGCTGGGTCGCTGCACACAAGTACTGCTTCCATCAAAGAACTGTCAATTCAGAAAACTGGAGTAACGTTAAGTTCCAGTTATT  3100

          ****************************************************************************************************
Reference TGTCGACAAAATTGAGTTCTACATCAGATATTACTATTGAACTTATTACTACTGAACTTATTACTACTGAACTTACTACTATCGAAGATAACGAACCAAA  3200
   Result TGTCGACAAAATTGAGTTCTACATCAGATATTACTATTGAACTTATTACTACTGAACTTATTACTACTGAACTTACTACTATCGAAGATAACGAACCAAA  3200

          ****************************************************************************************************
Reference CACTTTTACTTCAACTCCGTCAAGTCATTCTGAAATATTTTCTAGTGATAATAGTGTTTTATCAAAACAAGTTGATAGAGAAAGTACTATTAAAACTTCT  3300
   Result CACTTTTACTTCAACTCCGTCAAGTCATTCTGAAATATTTTCTAGTGATAATAGTGTTTTATCAAAACAAGTTGATAGAGAAAGTACTATTAAAACTTCT  3300

          ****************************************************************************************************
Reference CCTACAACGGACGTCACTACAGTATCATCTTTATCAGTACATTCAACAGAAGCTTCTACTGCAACACTCGGAGAAAATTCATTTAGCAATGTTGCCAGTA  3400
   Result CCTACAACGGACGTCACTACAGTATCATCTTTATCAGTACATTCAACAGAAGCTTCTACTGCAACACTCGGAGAAAATTCATTTAGCAATGTTGCCAGTA  3400

          ****************************************************************************************************
Reference CTCCACTGAATATTGCAACATCTTTAAGATCAACAAGTTCATCATCAAATCATGCTACCGAATCAAGTGGAACGGTTAAAAGTGAAGCAAGTGCAGAAAC  3500
   Result CTCCACTGAATATTGCAACATCTTTAAGATCAACAAGTTCATCATCAAATCATGCTACCGAATCAAGTGGAACGGTTAAAAGTGAAGCAAGTGCAGAAAC  3500

          ****************************************************************************************************
Reference AATTCCTTCTCCACCTACTTCAACTGACAACAGACTAAGCTATTCAACTGAAGAAGCCAAAGGTATTACATATGCTAATTCAGGTTCTACAAATAACCTC  3600
   Result AATTCCTTCTCCACCTACTTCAACTGACAACAGACTAAGCTATTCAACTGAAGAAGCCAAAGGTATTACATATGCTAATTCAGGTTCTACAAATAACCTC  3600

          ****************************************************************************************************
Reference ATAAACGAATCTCAAGTGGCCGCTCCAACTGATTCTACTTCAGTGTTGATTGAAAACCCAGTAGTAACTTCGACCTTTGATGATAACTCGTCAGCAGCTG  3700
   Result ATAAACGAATCTCAAGTGGCCGCTCCAACTGATTCTACTTCAGTGTTGATTGAAAACCCAGTAGTAACTTCGACCTTTGATGATAACTCGTCAGCAGCTG  3700

          ****************************************************************************************************
Reference TGGATCAACCAAGTAAAACTAAGTCGATTGAAGAATCTATTATGAATCCTGATTCAACCAATGAAACTAACAATGGATTTATCGCTACTTTATCACAAGC  3800
   Result TGGATCAACCAAGTAAAACTAAGTCGATTGAAGAATCTATTATGAATCCTGATTCAACCAATGAAACTAACAATGGATTTATCGCTACTTTATCACAAGC  3800

          ****************************************************************************************************
Reference TCAAGTACCAAGTTCCTCGATTCATTCAGAGTTAATACTGACTACGACGGCTAAAACAACTGATGCTTCGATGAATGGAGACAGTGCTGCTTCAAACTCA  3900
   Result TCAAGTACCAAGTTCCTCGATTCATTCAGAGTTAATACTGACTACGACGGCTAAAACAACTGATGCTTCGATGAATGGAGACAGTGCTGCTTCAAACTCA  3900

          ****************************************************************************************************
Reference CAGCCAACCACATTAATTCAACAAGTAGCAACTTCCTCCTACAATCAACCCCTTATTACCACTTATGCCGGATCTTCATCCGCCACTAAACATCCTTCCT  4000
   Result CAGCCAACCACATTAATTCAACAAGTAGCAACTTCCTCCTACAATCAACCCCTTATTACCACTTATGCCGGATCTTCATCCGCCACTAAACATCCTTCCT  4000
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Appendix IV

Sequencing results of *ALS6* inserted in plasmid.

Appendix IV (cont'd)

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          ****************************************************************************************************
Reference CGAGAACAGTCACTGATTCTACAACAGCAATTACCACATTACCGTTTGATCCCACAGTTGATAAAACCAAAACCATTGAAGTAATAGAACCCATCCCTAC 1100
   Result CGAGAACAGTCACTGATTCTACAACAGCAATTACCACATTACCGTTTGATCCCACAGTTGATAAAACCAAAACCATTGAAGTAATAGAACCCATCCCTAC 1100

          ****************************************************************************************************
Reference TACCACTATTACTACTTCATATGTTGGGATTTCTACTTCACTTTCTACGAAGACTGCAACTATTGGAGGAACAGCAACTGTTGTTGTTGATGTTCCCTAT 1200
   Result TACCACTATTACTACTTCATATGTTGGGATTTCTACTTCACTTTCTACGAAGACTGCAACTATTGGAGGAACAGCAACTGTTGTTGTTGATGTTCCCTAT 1200

          ****************************************************************************************************
Reference CATACAACTACCACTATCACTAGTATATGGACTGGATCAGCTACCACATCAAGTACTTATACAAATCCCACTGACTCGATTGATACAGTTGTTGTACAAG 1300
   Result CATACAACTACCACTATCACTAGTATATGGACTGGATCAGCTACCACATCAAGTACTTATACAAATCCCACTGACTCGATTGATACAGTTGTTGTACAAG 1300

          ****************************************************************************************************
Reference TTCCACTGCCAAATCCAACAGTTACAACTACTCAGTTTTGGTCAGGAAGTGTGCCCACAACCGAAACTGTGACCACTGGACCACAAGGAACGGATAGTGT 1400
   Result TTCCACTGCCAAATCCAACAGTTACAACTACTCAGTTTTGGTCAGGAAGTGTGCCCACAACCGAAACTGTGACCACTGGACCACAAGGAACGGATAGTGT 1400

          ****************************************************************************************************
Reference GATCATCAAGGAGCCACACAACCCTACTGTGACTACCACTGAGTTTTGGTCAGAATCATTTGCTACTACTGAGACAGTCACCAACGGTCCAGAAGGCACT 1500
   Result GATCATCAAGGAGCCACACAACCCTACTGTGACTACCACTGAGTTTTGGTCAGAATCATTTGCTACTACTGAGACAGTCACCAACGGTCCAGAAGGCACT 1500

          ****************************************************************************************************
Reference GACTCAGTCATTGTTAGAGAACCACACAATCCAACTGTGACAACAACCGAGTTTTGGTCAGAATCATTTGCTACTACTGAGACAGTCACCAACGGTCCAG 1600
   Result GACTCAGTCATTGTTAGAGAACCACACAATCCAACTGTGACAACAACCGAGTTTTGGTCAGAATCATTTGCTACTACTGAGACAGTCACCAACGGTCCAG 1600

          ****************************************************************************************************
Reference AAGGCACTGACTCAGTCATTGTTAGAGAACCACACAATCCAACTGTGACAACAACCGAGTTTTGGTCAGAATCATTTGCCACTACTGAAACGATAACTAC 1700
   Result AAGGCACTGACTCAGTCATTGTTAGAGAACCACACAATCCAACTGTGACAACAACCGAGTTTTGGTCAGAATCATTTGCCACTACTGAAACGATAACTAC 1700

          ****************************************************************************************************
Reference AGGGCCACTTGGCACTGATAGTATCGTTATACATGATCCATTGGAAGAACTGTCTTCTAGTACTGCTATTGAGTCAAGTGATTCTAATATTTCAAGCTCA 1800
   Result AGGGCCACTTGGCACTGATAGTATCGTTATACATGATCCATTGGAAGAACTGTCTTCTAGTACTGCTATTGAGTCAAGTGATTCTAATATTTCAAGCTCA 1800

          ****************************************************************************************************
Reference GCTCAAGAATCATCCAGTCTGGTTGAACAGTCATTGACTTCTGCTGACGAGACTTCAAGTATAGTTGAATTGTCATCAAGCTCAGACATTCCATCAAGCT 1900
   Result GCTCAAGAATCATCCAGTCTGGTTGAACAGTCATTGACTTCTGCTGACGAGACTTCAAGTATAGTTGAATTGTCATCAAGCTCAGACATTCCATCAAGCT 1900

          ****************************************************************************************************
Reference CAATTGGGTTAACATCTAGTGAGTCGTCTACTGTCTCAAGTTATGATAGCTACTCTTCAAGTACTAGCGAATCATCTATTGCTTCAAGTTATGATAGCTA 2000
   Result CAATTGGGTTAACATCTAGTGAGTCGTCTACTGTCTCAAGTTATGATAGCTACTCTTCAAGTACTAGCGAATCATCTATTGCTTCAAGTTATGATAGCTA 2000
```

Appendix IV (cont'd)

Appendix IV (cont'd)

Appendix IV (cont'd)

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          ****************************************************************************************************
Reference ATCAACCCCTTATTACTACTTATGCCGGATCTTCATCCGCCACTAAACATCCTTCCTGGTTGCTTAAATTTATTAGCGTTGCATTATTCTTCTTCTTATG   4100
   Result ATCAACCCCTTATTACTACTTATGCCGGATCTTCATCCGCCACTAAACATCCTTCCTGGTTGCTTAAATTTATTAGCGTTGCATTATTCTTCTTCTTATG   4100

          *
Reference A  4101
   Result A  4101
```